Oryza Vol. 52 No.1, 2015 (46-53)

NaCl induced changes in the ionic and osmotic components in rice cultivars vis-a-vis that in a natural halophyte

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ABSTRACT

Although accumulation of osmolytes like proline and glycinebetaine is well known phenomenon in plants under salt stress, the report on influence of salinity on accumulation osmolytes is scarce in halophytes, which grow in saline habitat. Hence, the present study was designed to address the same with aim to identify any commonness in the salt tolerance mechanism operative in glycophytes and halophytes considering salt-sensitive and -tolerant rice cultivars, Badami and Pokali, respectively as glycophytes and Suaeda maritima as halophyte. The study revealed accumulation of Na^+ in both the rice cultivars and S. maritima upon application of NaCl, but while NaCl promoted the growth of S. maritima, it inhibited the growth of the rice cultivars. However, the growth of Badami was inhibited more severely than that of Pokali. Estimation of accumulation of osmolytes, such as proline and quaternary ammonium compound (QAC) revealed that both the rice cultivars accumulated proline, but only Pokali accumulated QAC highly significantly. The accumulation of QAC was even greater in S. maritima. Expression analysis of the genes involved in synthesis of proline and glycinebetaine suggested the latter to be the most probable compound protecting Pokali and S. maritima against osmotic stress.

Key words: *NaCl salinity, Oryza sativa, Suaeda maritima, proline, glycinebetaine*

Abiotic stresses in the form of water deficit, high salinity or long period of drought have been the major selective forces in plant evolution (Inze and Van Montague, 1995), but at the same time constitute serious threats to agriculture. Soil salinity in particular is one of the major environmental stresses causing a significant loss of productivity in world agriculture, especially in irrigated lands (Zhu, 2001; Horie and Schroeder, 2004). Among the crop plants, rice (*Oryza sativa* L.), which is a staple crop for over 3 billion people globally, has low salt tolerance; its production and planting area are greatly affected by soil salinity (Akbar and Ponnamperuma, 1980). At the present, salinity is the second most widespread soil problem in rice growing countries after drought and is considered as a serious constraint to rice production worldwide (Kumar *et al*., 2015; Rao *et al*., 2013). Millions of hectares in the humid regions of South and Southeast Asia are technically suited for rice production, but are left uncultivated or are grown with very low yields because of salinity problem; 12 million ha of land area is thought to be salinity affected in Asia with India having >50% salinity affected area (Kumar *et al*., 2015).

The constituents of salinity are ionic in nature, represented by the cations of alkali (K^+ and Na^+) and alkaline earth (Ca^{2+} and Mg^{2+}) metals, while chloride forms the most abundant anion followed by SO_4^{2-} (Egan and Ungar 1998). In terrestrial environment, the relative levels of these cations and anions may vary greatly, but Na^+ and Cl^- remain the major contributors to salinity. In seawater Na^+ and Cl^- together constitute 2/3rd of the total salinity, while the presence of SO_4^{2-} is negligible in comparison to Cl^- (Parsons *et al*., 1984). Plants experiencing salt-stress suffers in two ways: 1) reduced water availability, and 2) accumulation of inorganic ions, mostly Na^+. Neither excess Na^+ nor insufficient water has single cellular target; while the deleterious effects of insufficient water results from dehydration, which can damage proteins and membrane, the deleterious

effect of excess ions results due to ion displacement in which the accumulating ions displaces inorganic ions in the proteins and membranes resulting in changes in their properties, which renders them non-functional, reduces their functional potential or alter their function per se (Garcia *et al*., 1997). Reduced water availability is, however, the most immediate effect of salt-stress in plants.

Regulation of ionic balance is also necessary for maintaining the normal metabolism, otherwise disturbed under saline condition. Plants experiencing salt stress show changes in the intracellular content of both inorganic ions and organic molecules necessary for osmotic adjustment. However, so far there has not been any concerted effort to look into salt-induced changes in all these components together and considering plants from diverse habitat. The present study was thus designed to look into changes in Na^+ and K^+ contents, the two important components of salinity, and the levels of proline and quaternary ammonium compounds (QAC), the two widely reported osmotic components considering salt-tolerant and non-tolerant cultivars of rice and a natural halophyte, *Suaeda maritima* with aim of identifying the salt tolerance mechanism operative in them.

MATERIALS AND METHODS

Seeds of *S. maritima* L. were collected from adult plants growing along the mangrove coastal belt in, Bhadrak (21.13° N, 86.76° E), Odisha, India. The seeds were spread on autoclaved soil in plastic pots having holes at the bottom and watered every day alternately with 1/ 10th Hoagland's solution or Milli-Q water. The seedlings were allowed to grow in a growth chamber maintained at 24 ± 3 °C, 70-75 % relative humidity and 14 h light (200 mmol m^{-2} s^{-1})/10 h dark cycle. After 3-4 weeks the seedlings were approximately 2 cm in height. At this stage, the seedlings were transferred to soil in plastic pots. The seedlings were set to acclimatize and grow for ~3 months under natural day/night cycle in a greenhouse maintained at 24 ± 3 °C and 70-75 % relative humidity. The individual pots were watered every day alternately with 1/10th Hoagland's solution or Milli-Q water except on the penultimate day of NaCl application. For NaCl application, initially 500 ml of 0.25 % NaCl prepared in 1/10th strength Hoagland's solution was poured into the individual pots early in the morning. The control pots received only 1/10th Hoagland's solution. After 30 min, 50 ml of 85, 255 and 425 mM of NaCl prepared in 1/10th strength Hoagland's solution was poured into the individual pots kept for salt application at regular interval. *O. sativa* cv. Pokali and *O. sativa* cv. Badami were used as the salt-tolerant and non-tolerant cultivars, respectively in the study. Their seeds were germinated on moist filter paper in petri-plates and the germinated seeds were grown on 1/10th Hoagland's solution in 200 ml beakers for 7 days in the green house under conditions mentioned above. Half an hour before switching on the light the seedlings were treated with 0.25 % NaCl, followed by increasing the concentration to 85, 255 and 425 mM. Aerial portion of the test plants was excised after the required exposure duration and preserved in liquid N_2 and stored at -80 °C until use. For proline estimation, the samples were weighed and preserved. Exposure was for 7 d for measurement of weight, 24 h for the estimation of ions and osmolytes, and 12 h for study of expression of the genes.

For estimation of Na^+ and K^+ contents, the harvested plant samples were wrapped in aluminum foil and dried in an oven for 24 h. The dried samples were weighed and digested separately in 5 ml of 2:1 nitric acid and perchloric acid in long corning tubes until the solution became clear. The digested samples were allowed to cool and then made-up volume using double distilled water. The concentrations of Na^+ and K^+ in the solutions were determined on an atomic Absorption Spectrophotometer (Analytik Jena).

The proline contents were determined following the procedure of Bates *et al*. (1973). Approximately 500 mg of individual fresh samples were homogenized separately in 3 % aqueous sulfosalicylic acid and the homogenates were centrifuged at 12000 x g. The reaction mixture consisting of 2 ml supernatant, 2 ml acid ninhydrin (1.2 g ninhydrin in 30 ml glacial acetic acid and 20 ml 6 M orthophosphoric acid) and 2 ml of glacial acetic acid was boiled at 100 °C for 1 h. The reaction was terminated over an ice-bath. The reaction mixture was extracted with 4 ml toluene, and absorbance of the toluene was read at 520 nm. The concentration of proline was determined from a standard graph and was expressed as mg g^{-1} fresh weight (FW).

QAC contents were determined following Greive and Grattan (1983). Finely ground dry plant

material (0.5 g) was mechanically shaken with 20 ml of deionized water for 48 h at 25 °C. The samples were then filtered and the filtrate was stored in freezer until analysis.Thawed extracts were diluted 1:1 with 2 N sulphuric acid. Aliquot (0.5 ml) was measured into test tube and cooled in ice water for 1 h. To the individual tubes was added 2 ml of cold potassium iodide-iodine reagent (15.7 g iodine and 20 g potassium iodide dissolved in 100 ml of water) and the mixture was gently mixed with vortex mixture. The samples were stored at 0-4 °C for 16 h. After this the samples were transferred to centrifuge tubes and then centrifuged at 10,000 x g for 15 minutes at 0 °C. The supernatant was carefully aspirated with 1 ml micropipette. The periodite crystals were dissolved in 9 ml of 1,2-dichloro ethane (reagent grade). After 2.0-2.5 h the absorbance was measured at 365 nm with UV-visible spectrophotometer. Reference standards of glycine-betaine (50-200 µg/ml) were prepared in 2 N sulphuric acid and the procedure for sample estimation was followed.

For the study of expression of various genes, total RNA was extracted from using TRIZOL (Invitrogen, USA) following the steps outlined in the manual. The individual RNA pellets obtained were suspended in DEPC-water. The quality and quantity of the individual RNA preparation was checked on agarose gel and a nano-drop spectrophotometer. The RNA preparations were stored at -80 °C until use. QuantiTect Reverse Transcription Kit (Qiagen), which provides optimized mix of oligo-dT and random primers and gDNA Wipeout Buffer, was used to convert RNA to cDNA. The cDNA so prepared was used for RT-qPCR. All reverse transcription reactions were carried out in a total volume of 20 ml each taking 1 mg gDNA free total RNA as per the protocol provided in the kit manual. Gene-specific primers of Tm not less than 59.0 °C and length not less than 20-mer were designed using Primer Blast software at NCBI site (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). QuantiTect SYBR Green PCR Kit (Qiagen) was used for the RT-qPCR on LightCycler® 480 Real-Time PCR System (Roche) and the reactions were individually run in 25 µl volume on 96 well-plate as detailed in the manual. 18S rRNA of each cDNA preparation served as reference gene. The relative levels of templates of the individual gene in each test plants were quantified following Pfaffl (2001) considering 18S rRNA templates in the sample as the reference level and presented as relative fold change in expression.

RESULTS AND DISCUSSION

In order to check if the test plants selected for the study really differed in their tolerance/sensitiveness to NaCl, their growth performance under NaCl application was measured. The growth performance was measured as change in dry wt. of the seedlings after application of NaCl for 7 days (Fig. 1). Among the rice cultivars, Badami was found to be much more sensitive to NaCl application than Pokali. The dry wt. of the individual seedlings of Badami was reduced by approximately 50 % compared to that of control value after 7 days of exposure to 85 mM NaCl. In contrast to Badami, there occurred a slight increase in the dry wt. of the seedlings of Pokali was less than 10 % of the control value in response to 85 mM NaCl treatment. At the other treatment concentrations also the growth performance of Pokali was better than Badami.

In contrast to the rice cultivars, *S. maritima* showed increase in dry wt. of the seedling upon exposure to NaCl; the dry wt. of the 255 mM NaCl treated seedlings was more than two times of the dry wt. of the control seedlings (Fig. 1). Application of 85 mM NaCl also resulted in approximately two times increase in dry wt. of the seedlings when compared to the control value. Thus, *S. maritima* was not only salt-tolerant; it also required NaCl for its growth. This was in contrast to the salt tolerant cultivar rice (Pokali), which showed no enhancement in growth in response to NaCl.

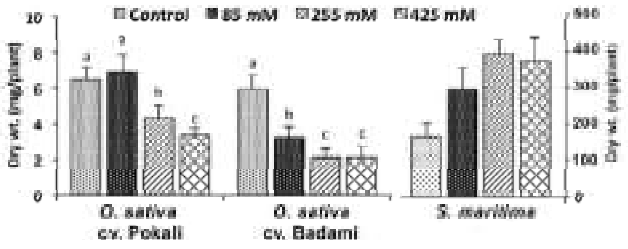

Fig. 1. Changes in dry weight of the test plants exposed to various concentrations of NaCl for seven days. Data are mean ±sd of five independent observations. The mean bars for a test plants marked with same alphabet do not differ significantly from each other.

The tissue content of only Na^+ and K^+ was measured considering the presence of Na^+ in large amount in saline soil and water and well known requirement of K^+ in the cells' physiology. The uptake of these ions in the leaf tissue by the test plants was measured at various exposure concentrations (Fig. 2). The Na^+ content of the leaf tissue increased significantly in all the three test plants upon their exposure to NaCl. The level of Na^+ was very low in the control plants of both the rice cultivars. The tissue concentration of Na^+ increased more than 600 ppb in both the rice cultivars upon exposure to 85 mM NaCl. At 255 mM treatment, however, the two cultivars differed greatly in accumulation of the ion; while in Badami the Na^+

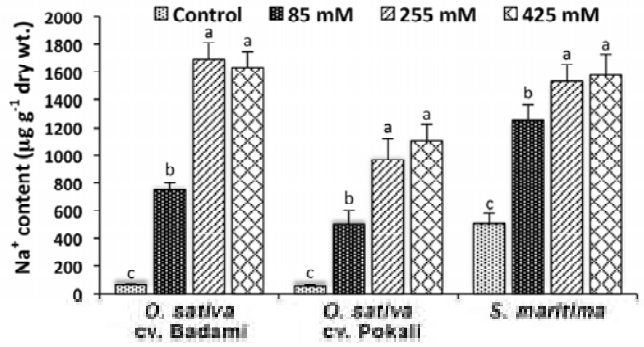

Fig. 2. Changes in Na^+ content of the test plants exposed to various concentrations of NaCl for 24 h. Other details as in Fig. 1.

content increased to more than 1600 ppb, Pokali showed Na^+ accumulation of less than 1100 ppb. Furthermore, in Badami there was no further increase in Na^+ content in the leaf tissue upon exposure of the plant to 425 mM NaCl, whereas the Na^+ content in Pokali did increase a little. Hence, the increase in Na^+ content in the leaves of Pokali was somewhat dependent on the NaCl treatment concentration, but it was not so in the case of Badami. Besides, Pokali showed much less accumulation of Na^+ in the leaf tissue when compared to Badami at all the treatment concentrations. In contrast to the rice cultivars, the control *S. maritima* plants showed a vey high Na^+ content in its leaf tissue, approximately 600 ppb, which was more or less equal to the level of Na^+ seen in the rice cultivars after their exposure to 85 mM NaCl. Besides, the plant (*S. maritima*) showed more or less similar accumulation of Na^+ in its leaf tissue at all the treatment concentrations, unlike the rice cultivars. Moreover, the accumulation of Na^+ in *S. maritima* at all the NaCl exposure concentrations was greater than that observed in the two rice cultivars.

Unlike that of Na^+, the concentration of K^+ in the leaves of the test plants decreased significantly upon their exposure to NaCl (Fig. 3). The decrease in the K^+ content was found to be NaCl treatment concentration dependent in both the rice cultivars, and the decrease was inversely related to the NaCl treatment concentrations. Compared to the rice cultivars, the K^+ content in the leaves of the control *S. maritima* was much less. The tissue content of the ion decreased further upon NaCl treatment. However, the decrease was not dependent on the NaCl treatment concentrations, unlike that in the rice cultivars. Furthermore, unlike the rice cultivars, the decrease in K^+ content in *S. maritima* was similar at all the NaCl treatment concentrations.

The tissue concentration of proline increased significantly in all the test plants in response to NaCl application (Fig. 4). The increase was much more significant and concentration dependent in the rice cultivars compared to that in *S. maritima*. Among the rice cultivars, Pokali showed greater accumulation of the osmoticum than Badami at all the treatment concentrations. The increase in the tissue concentration of proline in response to NaCl application was although significant in *S. maritima* at high application concentrations, the increase was approximately only two times in contrast to more than seven times increase observed in the rice cultivars.

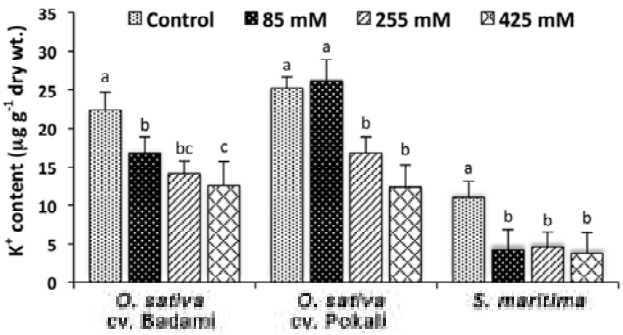

Fig. 3. Changes in K^+ content of the test plants exposed to various concentrations of NaCl for 24 h. Other details as in Fig. 1.

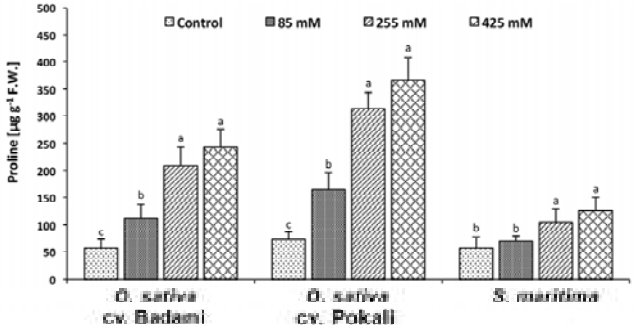

Fig. 4. Changes in proline content of the test plants exposed to various concentrations of NaCl for 24 h. Other details as in Fig. 1.

Unlike proline, QAC showed much accumulation in *S. maritima* compared to that in the rice cultivars in response to NaCl application (Fig. 5). The increase in accumulation of QAC was minimum in the salt-sensitive rice cultivar Badami, showing only 10-15 % increase irrespective of the NaCl application concentrations. The salt-tolerant Pokali, however, showed NaCl concentration dependent increase in accumulation of QAC, which increased to approximately three times. The increase in tissue concentration of QAC in *S. maritima* was, however, approximately five times the control level at 425 mM and was highly NaCl concentration dependent.

Figure 6 shows expression level of the genes involved in synthesis of proline and glycinebetaine (a QAC compound); the expression has been shown in fold change at 255 mM over control in 12 h exposure. Among the test plants, *S. maritima* showed the maximum response of the four genes involved in glycinebetaine synthesis. These were *BADH*

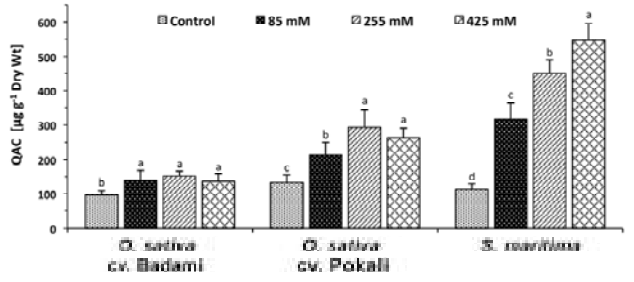

Fig. 5. Changes in quaternary ammonium compounds (QAC) content of the test plants exposed to various concentrations of NaCl for 24 h. Other details as in Fig. 1.

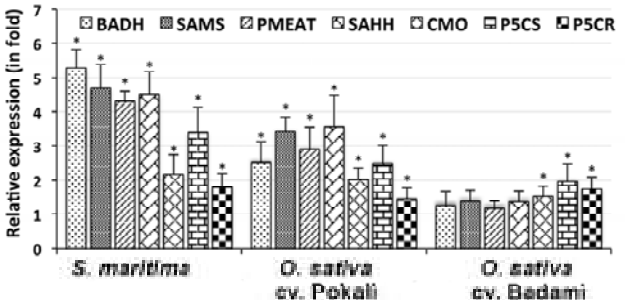

Fig. 6. Changes in expression (in fold) of betainealdehyde dehydrogenase (BADH), S-adnosylmethionine synthase (SAMS), phosphoethanolamine N-methyltransferase (PMEAT), S-adenosylhomocysteine hydrolase (SAHH), choline monoxygenase (CMO), Δ^1-pyrroline-5-carboxylate synthase (P5CS) and Δ^1-pyrroline-5-carboxylate reductase (P5CR) in the test plants exposed to 255 mM NaCl for 12 h over the control level. Data are mean ±sd of three independent observations.The individual mean bars marked by asterisk (*) differs significantly from the control level (considered as unity) at least at $p \leq 0.05$.

(betainealdehyde dehydrogenase), *SAMS* (S-adenosylmethionine synthase), *PMEAT* (phosphoethanolamine N-methyltransferase) and *SAHH* (S-adenosylhomocysteine hydrolase). Their expression increased by more than four fold. The increase in their expression was also significant in Pokali in response to NaCl application although comparatively less than that observed in *S. maritima*. In contrast to Pokali, Badami did not show significant change in expression of these genes in response to NaCl application. However, there did occur significant increase in the expression level of choline monoxygenase (*CMO*), which converts choline to betainealdehyde in glycinebetaine synthesis pathway. Pokali and *S. maritima* showed nearly two fold increase in its level in response to NaCl application. All the three test plants showed significant change in the expression of *P5CS* (Δ^1-pyrroline-5-carboxylate synthase) and *P5CR* (Δ^1-pyrroline-5-carboxylate reductase), the genes involved in proline synthesis. Increase in their expression was more or less similar in all the three plants.

Salt tolerance in plants varies greatly, as is observed in the present case as well. Among the cereals, rice has been reported to be the most salt-sensitive

(Munns and Tester, 2008). The salt sensitiveness of rice is also reflected from the present study showing approximately 50 % decrease in tissue dry wt. of one of the rice cultivars, *O. sativa* Badami upon exposure to 85 mM NaCl for seven days (Fig. 1). The results also indicated that the other cultivar, *O. sativa* cv. Pokali was tolerant to salinity to a great extent showing a slight increase in growth upon exposure to 85 mM NaCl for seven days. Unlike glycophytes like rice, the halophytes require high concentration of NaCl (100-200 mM) for optimum growth (Flowers *et al*. 1977). This is also reflected in the present study as the dry mass of *S. maritima* increased approximately 100 % upon receiving 85 mM NaCl, and by more than 100 % upon receiving 255 mM NaCl application (Fig. 1).

As the salinity components are ionic in nature, and cell membranes are highly selectively permeable to ions, salt tolerance/toxicity has generally been studied vis-à-vis the intracellular ionic build-up and their relative levels. In terms of ion relation, the specific symptoms of sodium toxicity in terrestrial plants include high tissue sodium concentrations and low K^+:Na^+ ratios (Maas and Grieve, 1987). However, in wheat salt sensitivity has been correlated with the sodium concentration in the shoot (Schachtman *et al*., 1989; Schachtman and Munns, 1992) and tolerance is considered to depend on the ability of the plant to exclude sodium from the shoot. In fact limited sodium uptake is considered to be a trait related to salinity tolerance in several crop plants (Fortmeier and Schuber, 1995; Gorham *et al*., 1990; Gregoria and Senadhira, 1993; Subbarao *et al*., 1990). This is also reflected from the analysis of Na^+ content of the two rice cultivars; the uptake of Na^+ by Pokali, the salt-tolerant cultivar was considerably less than that of Badami, the non-tolerant cultivar, at all the treatment concentrations (Fig. 2). In contrast accumulation of Na^+ did not affect the growth of *S. maritima*, as is universally accepted for halophytes (Flowers *et al*., 1977, Leidi and Sarz, 1997), indicating that during the course of evolution the halophytes have organized their physiological and metabolic processes in such a manner that uptake of Na^+ has became a necessity rather than a problem.

In contrast to Na^+ the intracellular level of K^+ decreased significantly in all the test species (Fig. 3). It is generally considered that salt tolerance in glycophytes is due to maintenance of high intracellular K^+ to Na^+ ratio through high K^+ selectivity (Alfocea *et al*., 1993). However, this was not reflected in the present study as the level of K^+ decreased in both Badami and Pokali with simultaneous increase in the intracellular level of Na^+ (Fig. 2, 3). Besides, *S. maritima* also showed highly significant decrease in the K^+ content despite being salt tolerant. Working on aquatic macrophytes, Rout and Shaw (2001) opined that it is the requirement of maintenance of a minimal K^+ level that is important rather the K^+ to Na^+ ratio for survival, which also appears to be true for *S. maritima* showing decrease in K^+ content (Fig. 3) despite maintaining good growth (Fig. 1).

Proline and glycinebetaine (QAC) are well known compatible osmolytes that accumulate in plants under osmotic stress (Hare and Cress, 1997; Jimenez-Bremon*et al*., 2006). Both proline and glycinebetaine have been reported to act as protective agents for cytoplasmic enzymes/proteins and as stabilizers of bio-membranes (Paleg *et al*., 1984; Kardpal and Rao, 1985; Zhao *et al*., 1992), besides functioning as osmolytes for osmotic adjustment under salt stress. So far as proline is concerned, the present study favours the view that the compound could act as an osmoprotectant in response to NaCl. But this seems to be mostly applicable mainly to glycophytes, as its level did increase highly significantly in *S. maritima* (Fig. 4). However, highly significant increase in its level in the salt-sensitive rice cultivar Badami (Fig. 4) and at the same time unable to perform well in terms of growth (Fig. 1) hardly supports any role of the compound in salt tolerance. In contrast, glycinebetaine appears to be the more likely osmoprotectant as its level increased highly significantly in *S. maritima*, and also greatly in the salt tolerant rice cultivar Pokali, but not to a great level in the salt-sensitive rice cultivar Badami although the increase was statistically significant (Fig. 5). Significant accumulation of glycinebetaine has also been reported in a terrestrial halophyte when transferred from non-saline to saline condition (Khan *et al*. 1998).

Highly significant accumulation of glycinebetaine in *S. maritima* is also reflected from highly significant increase in expression of the genes involved in synthesis of glycinebetaine, like *SAMS* that convert methionine to S-adenosylmethionine (SAM) required for donating methyl group in the reactions leading to synthesis of choline, *SAHH* that metabolizes

S-adenosyl-L-homocysteine (SAH) formed during the transmethylation reactions involving SAM and is inhibitory to these reactions, *PEAMT* that converts phosphatidyl-ethanolamine to choline, *CMO* that converts choline to betainealdehyde, and *BADH* that converts betainealdehyde to glycinebetaine. Significantly greater accumulation of QAC (Fig. 5) and greater expression of the genes involved in glycinebetaine synthesis (Fig. 6) in the salt-tolerant Pokali than in the salt-sensitive Badami is also suggestive of the possible involvement of glycinebetiane rather than proline in greater salt tolerance of the former than of the latter. As proline increased more or less to similar level in both the rice cultivars (Fig. 4), its role in salt-tolerance is questionable. Besides, the increase in the level of proline could be a result of mechanism other than synthesis as the expression of the genes involved in synthesis of proline, i.e. *P5CS* that converts glutamate to glutamic acid-5-semialdehyde, and *P5CR* that converts Δ^1-pyrroline-5-carboxylate (P5C) formed from spontaneous cyclization of GSA to proline.

The study thus revealed that different salt tolerance mechanisms are operative in rice, a glycophyte, and *S. maritima*, a halophyte, and that salt accumulation is not a reflection of toxicity and poor growth. It is the Na^+ sequestration and the osmoprotectant that determine the salt tolerance of plants. And the present study shows that increasing the synthesis and accumulation of glycinebetaine may increase salt tolerance in rice plant, highly desired for cultivating crop plant in salt-affected lands.

ACKNOWLEDGEMENT

The authors are thankful to the Director, Institute of Life Sciences for providing the necessary laboratory facility and financial assistance for the work. SAG thankfully acknowledges DBT for fellowship.

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