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Real time PCR expression analysis of gene encoding p5cs enzyme and proline metabolism under NaCl salinity in rice

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Abstract

Regulation of proline accumulation in seedlings of rice (*Oryza sativa* L. cv. Lunishree) was investigated. The increasing concentration of NaCl from 85 mM to 425 mM NaCl progressively increased proline content in rice. The maximum increase in proline content was recorded at 425 mM NaCl concentration as compared to control and other concentrations of NaCl. The highest significant activity of proline synthesizing enzymes, Δ^1-Pyrrolline-5-carboxylate synthetase, Δ^1-Pyrrolline-5-carboxylate reductase and Ornithine-δ-aminotransferase with lowest activity of proline hydrolysis enzymes;Proline dehydrogenase was also recorded at 425 mM NaCl salinity over control and other concentrations of NaCl with insignificant increase in the activity of Δ^1-Pyrrolline-5-carboxylate synthetase and Ornithine-δ-aminotransferase at 85 mM NaCl over control.It was found that the transcript of gene encoded with p5cs is up regulated about 1.35 folds under salinity stress. This gene synthesis an osmo protectant to help the plant resist the change in osmotic imbalances. Externally addition of $MnCl_2$ at 300mg/220 ml ½ strength Hoagland solution, having 1% NaCl, was also seen to increase 893.9 % proline content of this variety as compared to control.

Key words

OAT, P-5-CR, P-5-CS gene, P-5-CS, PDH, Proline, Rice, RT-PCR

Introduction

Rice (*Oryza sativa* L.) is the most important food crop of the developing world.Asian farmers produce about 90 % of the total, with two countries, China and India, growing more than half the total crop (USDA, 2008).Plants are exposed to various types of environmental stress.Soil salinity has been intensified more by global climate change due to the increased temperature in the future. There is the urgent need to identify and use salinity tolerant plants. Since plants can't move, many have evolved sophisticated mechanisms to adapt to undesirable environments. Osmotic regulation is an important mechanism for plant cellular homeostasis in saline conditions. Under salt stress, plants accumulate several compatible solutes in the cytosol, such as polyols, betaine, trehalsose, ectoine, proline and others (Hasegawa *et al*., 2000). Proline accumulation in plant cells exposed to salt or water stress is a widespread phenomenon (Lutts *et al*., 1999; Wang *et al*., 2011). However, the actual role of proline accumulation remains unclear (Rhodes *et al*., 1999) but it has been speculated that it can serve as an osmotic regulator, a protector of enzyme denaturation, a stabilizer of some macro molecules or molecular assemblies, are reservoir of nitrogen and carbon sources or a hydroxyl radical scavenger. However, some reports indicate no correlation between proline accumulation and stress resistance (Lutts *et al*., 1996 and Turan *et al.,* 2009).

In plants, proline is synthesized from glutamic acid (Glu) via Δ^1-pyrroline-5-carboxylate (p5c) by two enzymes,Δ^1-Pyrrolline-5-carboxylate synthetase, Δ^1-Pyrrolline-5-carboxylate reductase. It has been shown from labelling experiments that ornithine (Orn) can also serve as a precursor to proline biosynthesis in higher plants (Chiang and Dandekar, 1995). Proline is metabolized to glutamic acid via p5c by two enzymes, proline dehydrogenase and P5C dehydrogenase (Mattioni *et al*., 1997; Bakht *et al*., 2012). Besides proline feed back control of Δ^1-Pyrrolline-5-carboxylate synthetase actively, expression of Δ^1-Pyrrolline-5-carboxylate synthetase gene involves a second key level of regulation. Indeed Δ^1-Pyrrolline-5-carboxylate synthetase

gene is transcriptionally induced by osmotic stress and its induction precedes accumulation of proline. Accumulations of proline and expression of Δ^1-Pyrrolline-5-carboxylate synthetase gene is strongly induced after 12 hr in salt tolerant rice Lunishree under high salt conditions when compared with salt sensitive rice (Azzami *et al*., 2009). This strongly supports that proline accumulation and induction of Δ^1-Pyrrolline-5-carboxylate synthetase, shortly after the start of salt stress may be related to the degree of salt tolerance in *Oryza sativa* L. cv. Lunishree. In this regard, the present investigation was undertaken to know the role of these enzymes involved in proline metabolism with Δ^1-Pyrrolline-5-carboxylate synthetase gene under NaCl stress. The role of $MnCl_2$ in proline accumulation was also newly investigated to provide stress tolerance in this cultivar of rice seedlings.

Materials and Methods

Plant materials and growth conditions: This experiment was conducted in plastic glass of 250 ml capacity in growth chamber during *Kharif* season of 2009 at Institute of Life Science, Bhubaneswar, Orissa. Rice salt tolerant cv. Lunishree was used in this study. Seeds of Lunishree were obtained from Orissa University of Agriculture and Technology, Bhubaneswar. The surface-sterilized seeds were soaked in de-ionized (MilliQ) water overnight, transferred over wet filter paper in a Petriplate and kept at 25 °C for germination. Rice seeds germinated fully in 24 hr. Germinated rice seeds were grown (in a growth chamber) hydroponically over nylon nets in 250 ml capacity plastic glass containing half-strength Hoagland's solution. After 6 days, the seedlings in individual glass were treated with 85 mM, 255 mM and 425 mM NaCl. For this, initially NaCl sufficient to make the concentration of NaCl was dissolved in few ml of half-strength Hoagland's solution and was poured into individual beakers except for control. After incubation for 1 hr in dark, NaCl was added further to raise its concentration to desired the levels. Volume of the solution was maintained at 200 ml mark by adding half-strength Hoagland's solution. Plants were kept in dark for another hour after application of final treatment and then exposed to light (200 nmol m^{-2} s^{-1}). The growth chamber was maintained at 24±3 °C, relative humidity 70-75% with 14 hr light and 10 hr dark periodicity. The seedlings were harvested after 12 hr of treatment for enzyme activity study.

Determination of proline and protein: Leaves of seedlings were homogenized with 3% sulphosalicylic acid and the homogenate was centrifuged at 3,000 g for 20 min. The supernatant was treated with acetic acid and acid ninhydrin, boiled for 1 hr. and then absorbance at 520nm was determined. Contents of proline were expressed as µg g^{-1} f. wt. of leaf (Bates, *et al* ., 1973). Protein content was determined by the method of Bradford (1976) and expressed as mg g^{-1} f. wt. of leaf.

Enzyme assays: A similar extraction procedure was used for Δ^1-Pyrrolline-5-carboxylate synthetase and Proline dehydrogenase, which was mainly based on the procedure described by Lutts *et al*. (1999). Seedlings were homogenized in a prechilled mortar and pestle with 50 mM Tris-HCl buffer (pH 7.4) containing 7 mM $MgCl_2$, 0.6 M KCl and 3 mM EDTA. The homogenate was centrifuged at 15,000 *g* for 20 min. The solution used for extraction of Ornithine-δ-aminotransferase (OAT) was 100 mM potassium phosphate buffer (pH 7.4) containing 1 mM pyridoxal-5- phosphate, 1 mM EDTA and 10 mM 2-mercaptoethanol.The extract was centrifuged at 12,000 *g*. All the extraction procedures were conducted at 4°C. Δ^1-Pyrrolline-5-carboxylate reductase was assayed by NADH dependent reaction (Madan *et al*. 1995).The assay mixture contained 0.06 mM NADH, 0.15 mM P5C, 120 mM potassium phosphate buffer, 2 mM dithiothreitol, and the enzyme extract. The reaction was started by addition of P5C and decrease in absorbance was followed at 340 nm. Δ^1-Pyrrolline-5-carboxylate reductase was expressed as units mg^{-1} protein (1U=Amount of enzyme required to produce 1µ mol NADH per minute. Ornithine-δ-aminotransferase activity was assayed according to Vogel and Kopac (1960).The assay mixture contained 0.2 ml enzyme extract and 0.8 ml 100 mM potassium phosphate buffer (pH 8.0) containing 50 mM L-ornithine, 20 mM α-ketoglutarate and 1 mM pyridoxal-5-phosphate.The reaction medium was incubated at 37 °C for 30 min. The reaction was stopped by adding 0.5 ml trichloroacetic acid (10%) and colour was developed by incubating the reaction mixture with 0.5 ml *o*-aminobenzaldehyde (0.5%) in ethanol (95%) for 1 hour.After centrifugation at 12,000 *g* for 10 min, clear supernatant fraction was collected to measure the absorbance at 440 nm. Ornithine-δ-aminotransferase was expressed as units mg^{-1} protein (1U=Amount of enzyme required to produce 1n mol p5c per minute). Proline dehydrogenase activity was assayed by following NAD^+ reduction at 340 nm in a 0.15 M Na_2CO_3-HCl buffer (pH 10.3) containing 13 mM L-proline and 1.5 mM NAD^+ (Lutts *et al*., 1999) and expressed as units mg^{-1} protein (1U=Amount of enzyme required to produce 1µ mol NADH per minute).

RNA isolation : Plant tissue was ground in liquid nitrogen and total RNA was prepared using Trizol (Invitrogen).DNA contamination was eliminated by DNase treatment. RNA was quantified by UV, precipitated, resuspended in DEPC treated water in a concentration of 2 µg $µl^{-1}$ and stored at -80 ° C. RNA from control and treated plant were treated by adding required amount of 5X loading dye to each tube the sample was loaded to the agarose gel and electrophoresis was carried out by 5-6 volt cm^{-1}. RNA bands were visualized under UV light trans illuminator and photographed.

First strand cDNA synthesis : cDNA synthesis was performed for control as well as well as treated RNA sample. Molany Muranie Leukemia virus Reverse transcriptase is an RNA dependent DNA polymerase that can be used in cDNA synthesis with long messenger RNA template (>5 kb) because Rnase H activity of M-MLV RT is weaker than commonly used Avian myeloblastosis

virus (AMV) reverse transcriptase. In a sterile RNAse free micro centrifuge tube, the following components were added per reaction as nuclease free H_2O: 8µl; RNA: 2µ; oligo dT: 2µ; Random primer mixture: 2µ. It was then mixed gentlly and incubated at 72º for 2min and cooled for 2 min in ice. The following reagents were added to annealed primer/template in the order shown: dNTPs: 5µl; Buffer: 5µl; Reverse transcriptase: 1µl and mixed gentle by flicking the tube and incubated for 90 min at 42ºc in PCR machine. The first strand of cDNA was received. After synthesizing cDNA, the reaction tube was kept at -20 º C for gene synthesis.

Test for equal loading by giving 18s rRNA : PCR was done using 18srRNA primer in a microfuge tube. The reagents were added in control as well as treated cDNA as Nuclease free H_2O: 8µl; PCR buffer, 4µl; Mg: 3.5µl; 18s forward primer: 1µl; Reverse: 1µl; dNTPs: 1µl; Taq pol: 1µl; cDNA:1µl.

Gene Specific PCR amplification : Polymerase chain reaction was done using gene specific primer (p5cs F-5'ATTCCTCGTAA TGTTGGTG3' and R-'AGTGCAGGCCA TAGAACTA3').

In a micro fuse tube, reagents were added as Nuclease free H_2O: 8µl; PCR buffer: 4µl; Mg: 3.5µl; P5cs F: 1µl; P5csR: 1µl; DNTPs: 1µl; Taq pol: 5µl; cDNA: 2µl. PCR programmer was fixed as mentioned: Step-1:94ºC, Step-2:94ºC, Step-3: 51ºC, Step-4:72ºC, Step-5: back to step 2 followed by step 3 and step 4 for 35 cycles in a sequential manner, Step6-:72^0 c for 10 min, Step-7:4ºC for overnight.

Agarose gel electrophoresis: 2.0% agarose gel (TAE) was prepared with required ethidium bromide i.e. RNA bands of the leaf sample of *Oryza sativa* cv. Lunishree, 3 gm of agarose gel was dissolved in 25ml of ethidium bromide and PCR products were checked by agarose gel electrophoresis along.

Statistical analysis : All data were analyzed with three replication for analysis of variance by CRD method and standard error of each mean value was also calculated for presentation with bar diagram.

Results and Discussion

Proline content was found to be maximum at 425 mM NaCl concentration (160.15% more than control) followed by 255 mM NaCl concentration (106.94% more than control), 85 mM NaCl concentration (31.98 % more than control). Whereas, increase observed in proline content at 85 mM NaCl concentration was found insignificant (Table 1).The increase in proline content in plants under salt stress was due to hypothesis: that in plants proline is synthesized from glutamic acid (Glu) via Pyrroline-5-carboxylate (p5c) by two enzymes, Δ^1-Pyrroline-5-carboxylate synthetase, Δ^1-Pyrroline-5-carboxylate.It has been shown from labeling experiment that ornothin (orn) can also serve as a precursor to proline biosynthesis in higher plants (Chiang and Dardekar,1995). Isolation of cDNA encoding Ornithine-δ-aminotransferase in higher plants (Delauney and Verma, 1993: Roosens *et al*., 1998) suggests that Ornithine-δ-aminotransferase participates in proline biosynthesis by reducing p5c from ornithin and α-ketoglutarate. Most of the suggestions regarding tolerance of proline salinity in plants is based on its enhanced accumulation in response to stress. Proline accumulation in plant tissues has been suggested to result from decrease in proline degradation, increase in proline biosynthesis, decrease in protein synthesis or proline utilization, and hydrolysis of protein (Charest and Phan, 1999; Yoshiba *et al*., 1997).

The activity of Δ^1-Pyrroline-5-carboxylate synthetase in control leaf sample was recorded 0.2116 U mg^{-1} protein, which increased 6.21% at 85 mM NaCl concentration, 12.25 % at 255 mM NaCl concentration and 18.8 % at 425 mM NaCl concentration significantly. The maximum increase in Δ^1-Pyrroline-5-carboxylate synthetase activity was recorded at 425 mM NaCl concentration (Fig.1). Similar results have also been reported by Rout and Shaw, 1998.

The back ground (control level) activity of Δ^1-Pyrroline-5-carboxylate reductase was observed 0.1736 U mg^{-1} protein min^{-1}. A significant increase in Δ^1-Pyrroline-5-carboxylate reductase activity was noticed with increasing NaCl concentration up to 425

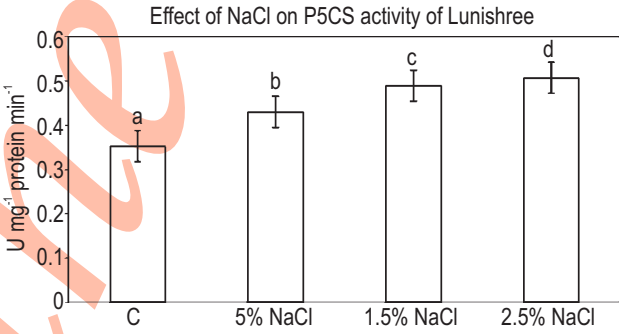

Fig. 1 : Effect of salinity levels on P5CS activity in Lunishree. Vertical bars indicate ± S.E.of mean followed by different alphabets are significantly different at P=0.05 for treatments. Mean are of three values

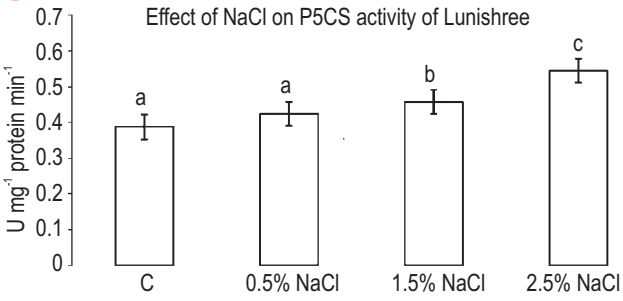

Fig. 2 : Effect of salinity levels on P5CR activity in Lunishree. Vertical bars indicate ±SE of mean followed by different alphabets are significantly different at P=0.05 for treatments. Mean are of three values

mM NaCl concentration (Fig. 2). The maximum increase in Δ^1-Pyrrolline-5-carboxylate reductase activity was recorded at 425 mM NaCl concentration (0.238 U mg^{-1} protein min^{-1}). 37.04 % more than control followed by 0.2243 U mg^{-1} protein min^{-1} at 255 mM NaCl concentration (29.17 % more than control) 0.202 U mg^{-1} protein min^{-1} at 85 mM NaCl concentration (16.31 % more than control). Although, the increase observed at 85 mM NaCl concentration was in-significant. Chuan chi Lin *et al*. (2002) reported similar results.

Similar to Δ^1-Pyrrolline-5-carboxylate synthetase, the back ground (control level) activity of Ornithine-δ–amino transferase was also noticed 0.2569 U mg^{-1} protein min^{-1}. A significantly improving trend was seen in Ornithine- δ-aminotransferase activity up to 425 mM NaCl concentration except 85 mM NaCl concentration where a non significant increase in Ornithine- δ-aminotransferase activity was recorded (Fig.3). A 48.02 % more activity of Ornithine-δ-aminotransferase was observed at 425 mM NaCl concentration than the back ground (control level) activity of Ornithine-δ-aminotransferase followed by 30.64 % more Ornithine-δ-aminotransferase activity at 255 mM NaCl concentration and 20.91 % more Ornithine-δ-aminotransferase activity at 85 mM NaCl concentration. Similar results were also reported by Lutts *et al*. (1996); Madan *et al*. (1995); Turan *et al*. (2009) and Bakht *et al*. (2012). The trend of increase in enzyme activity of Δ^1-Pyrrolline-5-carboxylate synthetase activity was recorded more than Δ^1-Pyrrolline-5-carboxylate reductase and Ornithine-δ-aminotransferase activity.

The activity of proline dehydrogenase at control level was 0.3346 U mg^{-1} protein min^{-1}. A significant decrease was recorded in proline dehydrogenase activity with an increasing level of NaCl concentration up to 425 mM (Fig.4). The maximum decrease in proline dehydrogenase activity was recorded at 425 mM NaCl concentration (0.232 U mg^{-1} protein min^{-1}), 30.67 % less than control followed by 0.251 U mg^{-1} protein min^{-1}. at 255 mM NaCl concentration (25.0 % less than control) and 0.279 U mg^{-1} protein min^{-1} at 85 mM NaCl concentration(16.63 % less than control). Similar reports were also given by Mattioni *et al*. (1997); Chuan *et al*. (2002). Although, the increase observed in proline dehydrogenase activity at 85 mM NaCl concentration was found in significant.

It is concluded that there same amount of RNA was present in both control as well as treated plant samples. An increase in the intensity in amplicon corresponding to Δ^1-Pyrrolline-5-carboxylate synthetase was visible where as amplicon corresponding to 18s was more or less same in intensity suggesting that equal amount of single strand cDNA was taken for RT-PCR. It was found that transcript of Δ^1-Pyrrolline-5-carboxylate synthetase was up regulated about 1.35 folds under salinity stress (Azzami *et al*., 2009).This gene synthesize proline as an osmoprotectant to help the plant resist changes in osmotic imbalance. A strong correlation between proline accumulation and the synthesis of transcript in leaves of rice plant under salinity stress. Reports claim about the use of this up regulated genes to be used as a protectant transgene for use in transgenic plant

Table 1 : Effect of NaCl salinity on proline content of rice cv Lunishree, grown in ½ strength Hoagland solution

Treatment	Proline (µg g^{-1} f.wt. leaf) at 12 HAT
Control	25.30 ± 2.41
0.5% NaCl	34.07 ± 2.12
1.5% NaCl	37.56 ± 1.98
2.5% NaCl	49.13 ± 2.06

HAT=Hours after treatment

Table 2 : Proline accumulation by adding ornithin (precursor of proline synthesis) with and without NaCl in ½ strength 220 ml Hoagland solution

Treatment	Proline content (µg g^{-1} f.wt. leaf) at 1 DAT
Control	1.86 ± 0.51
Control+75 m M Ornithin	13.08 ± 0.41
NaCl 250 mM	3.573 ± 0.32
NaCl 250 Mm+75 m M Ornithin	18.37 ± 0.49

DAT=Days after treatment

Table 3 : How to increase proline accumulation in plants under NaCl salt stress condition by adding different inorganic salts in ½ strength 220 ml Hoagland solution having 1.0 % NaCl

Treatment	Proline content (µg g^{-1} f.wt. leaf)
Control	50.43 ± 2.42
$CaCl_2$ 200 mg	71.20 ± 2.12
$CaCl_2$ 500 mg	94.13 ± 1.74
kCl 200 mg	103.83 ± 1.59
KCl 400 mg	81.10 ± 2.12
$MnCl_2$ 300 mg	341.10 ± 1.98
$MnCl_2$ 600 mg	67.83 ± 2.06
NH_4Cl_2 200 mg	73.86 ± 2.32
NH_4Cl_2 400 mg	61.3 ± 1.49

Table 4 : Proline accumulation in plants under NaCl salt stress condition by adding more effective different concentration of $MnCl_2$ in ½ strength 220 ml Hoagland solution having 1.0 % NaCl

Treatment	Proline content (µg g^{-1} f.wt. leaf) at 1 DAT
Control	34.5 ± 1.96
$MnCl_2$ 50 mg	66.0 ± 1.47
$MnCl_2$ 100 mg	99.03 ± 1.82
$MnCl_2$ 200 mg	182.4 ± 1.18
$MnCl_2$ 300 mg	342.9 ± 1.2
$MnCl_2$ 400 mg	175.6 ± 1.52
$MnCl_2$ 600 mg	97.43 ± 1.81

DAT=Days after treatment

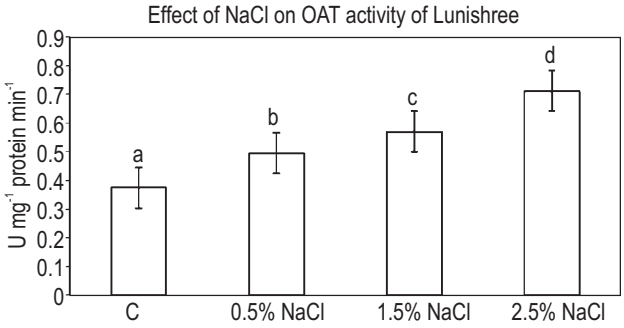

Fig. 3 : Effect of salinity levels on OAT activity in Lunishree. Vertical bars indicate ±SE of mean followed by different alphabets are significantly different at P=0.05 for treatments. Mean are of three values.

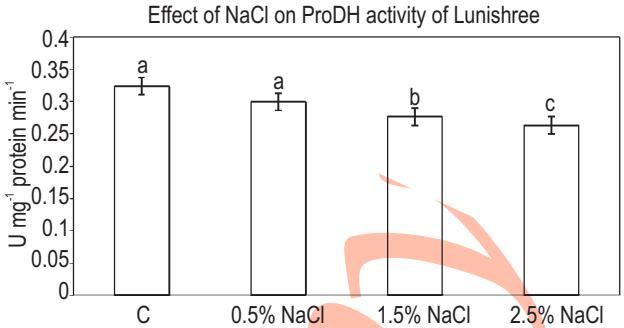

Fig. 4 : Effect of salinity levels on PDH activity in Lunishree. Vertical bars indicate ±SE of mean followed by different alphabets are significantly different at P=0.05 for treatments.Mean are of three values

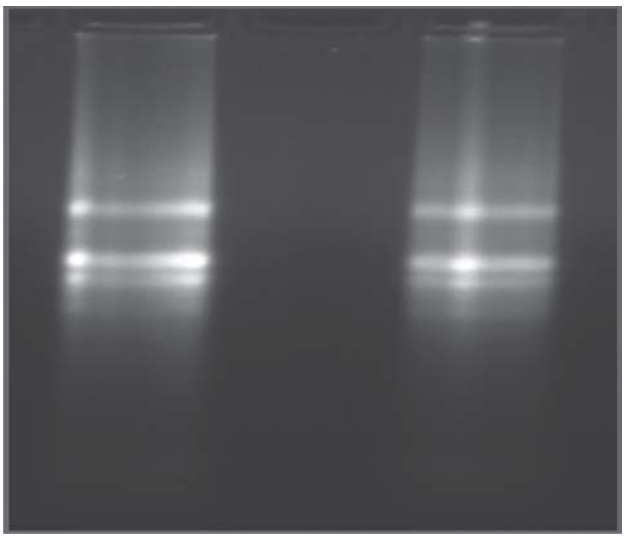

Fig. 5 : RNA bands of the leaf sample of *Oryza sativa* L cv. Lunishree

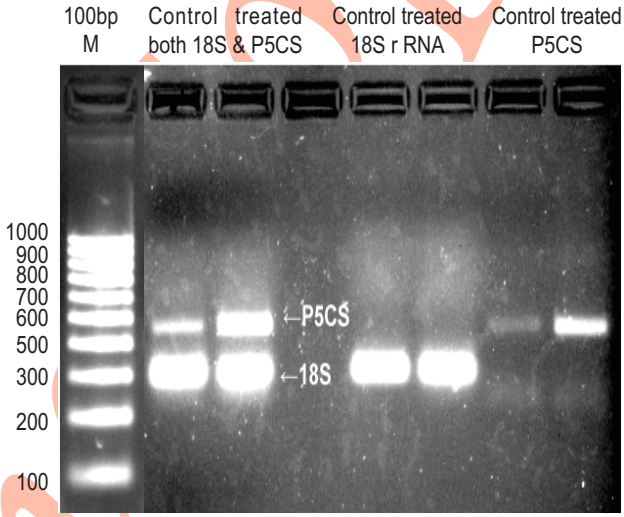

Fig. 6 : RT-PCR of single stranded DNA amplified from both control and salt treated (2.0 % NaCl) leaf sample of *oryza.sativa* L cv. lunisharee

regulation. This gene under strong promoter (35s AMV promoter) can be synthesized with the help of helper DNA and transferred to *Agrobacterium* strain, followed by co-cultivation with tissue of a crop plant and plant to be made transgenic (Fig. 5, 6 and7). This plant can be tested for salinity tolerance capacity generated by transgenic (Δ^1-Pyrrolline-5-carboxylate synthetase gene). The transcript of Δ^1-Pyrrolline-5-carboxylate synthetase gene was noticed 58 % under NaCl treated plants than control (43 %) Fig. 7.

In the present study, proline accumulation although increased significantly at all the treatment over control (Table 2). Ornithin is a precursor of proline biosynthesis, by using 75 mM ornithin in ½ strength Hoagland solution, 13.08 µg g^{-1} proline was recorded (603.2% more than control) whether getting 18.37 µg g^{-1} proline by 75 mM ornithin in 250 mM NaCl stress ((887.6 % more than control) so it is clearly seen by data that NaCl was positively involved in proline biosynthesis.

It was observed that NaCl induced proline biosynthesis in rice cv. lunishree. Thus it is of great interest to know whether proline accumulation was due to NaCl or due to other inorganic salts. To test this possibility, the effect of 200,400 mg $CaCl_2$; 200,400 mg KCl; 300,600 mg $MnCl_2$ and 200,400 mg NH_4Cl_2 in 220 ml ½ strength Hoagland solution having 1 % NaCl on rice cv. lunishree seedlings was determined. Results (Table 2) show that 300 mg increased highest proline content as compared to other concentrations and salts over control. This increase in proline content was 576.38 % more at of 300 mg $MnCl_2$ concentration followed by 500 mg $CaCl_2$ (86.65 %), 200 mg KCl (105.88 %), 200 mg NH_4Cl_2 (46.46%), 400 mg KCl (60.81 %), 600 mg $MnCl_2$ (34.5%) and 200 mg $CaCl_2$ (41.18 %) over control, respectively.

So it was important to study the effect of $MnCl_2$ on proline accumulation at low concentration also, for that a experiment was also carried out at lower concentration of $MnCl_2$ in 220 ml ½

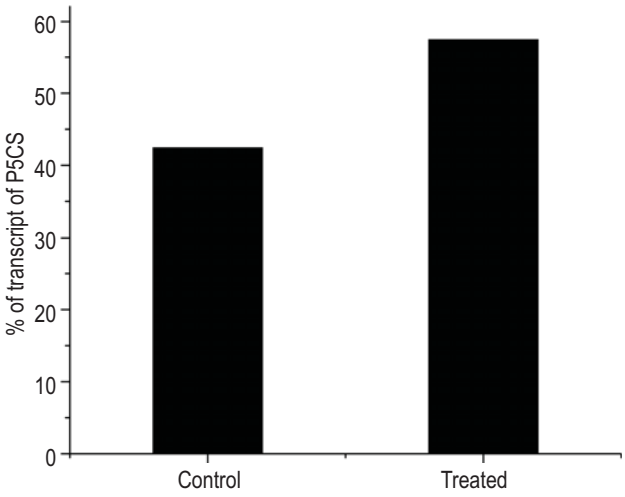

Fig. 7 : Percent (%) of transcript of p5cs

strength Hoagland solution having 1 % NaCl salt. Results (Table 3) indicated that a linear decrease in proline content was recorded in plants down to 50 mg concentration of $MnCl_2$ via 200 mg $MnCl_2$ and 100 mg $MnCl_2$ significantly. The results proved that 300 mg $MnCl_2$ concentration was highly contributing in increasing proline accumulation in rice cv lunishree under 1 % NaCl stress condition. Most likely synthesis of proline and its consequent accumulation in lunishree is a consequence of intracellular ionic adjustment that took place under salt stress to keep the metabolic activities going on.

Addition of $MnCl_2$ enhanced proline content in plants under 1 % NaCl stress. Thus, the role of proline as osmotic regulator; protector of enzyme denaturation; reservoir of nitrogen and carbon source for post growth; stabilizer of membranes and machinery of protein synthesis; hydroxyl radical scavenger and to provide salinity tolerance to plants may be promoted by addition of $MnCl_2$ under 1 % salt stress condition.

In conclusion, the effect of NaCl stress on enzyme activity involved in proline metabolism in rice could provide valuable information on the physiological significance of its accumulation. However, the results of this experiment provide more basic information, which would be valuable for future work in real time PCR expression analysis of genes encoding these Δ^1-Pyrrolline-5-carboxylate synthetase, Δ^1-Pyrrolline-5-carboxylate reductase and Ornithine-δ-aminotransferase enzymes of proline synthesis for developing transgenic plants in relation to salinity tolerance in rice. Externally, addition of $MnCl_2$ (@1.3 g l^{-1} of water as foliar spray) was also found to increase proline content in this variety under 1 % salt stress.

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References

Azzami, C.R., S. Edris and A.A. Mansour: Changes in wheat *P5CS* gene expression in response to Salt stress in wheat. *Egypt. J. Genet Cytol*., **38**, 375-368 (2009).

Bakht, J., J.K. Mohammad, A.K. Mohammad and S. Mohammad: Effect of salinity and ABA application on praline production and yield in wheat genotypes. *Pak. J. Bot*., **44**, 873-878 (2012).

Bates, L.S., S.P. Waldren and I.D. Teare: Rapid determination of free proline for water-stress studies. *Plant Soil*, **39**, 205–207 (1973).

Boyer, J.S.: Plant productivity and environment. *Sci.,* **218**, 443-448 (1982).

Bradford, M.M.: A rapid and sensitive method for the quantization of microgram Quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254 (1976).

Charest, C. and C.T. Phan: Cold acclimation of wheat (*Triticum aestivum* L): Properties of enzymes involved in proline metabolism. *Physiol. Plant.*, **80**, 159-168 (1990).

Chiang, H.H. and A.M. Dandekar: Regulation of proline accumulation in Arabidopsis thaliana (L.) Heynh during development and in response to desiccation. *Plant Cell Environ.*, **18**, 1280–1290 (1995).

Chuan, C.L., Y.T. Hsu and C.H. Kao: The effect of NaCl on proline accumulation in rice Leaves. *Plant Growth Regul.,* **36,** 275–285 (2002).

Hasegawa P.M., R.A. Bressan., J.K. Zhu and H.J. Bohnert: Plant cellular and molecular responses to high salinity. *Annual Review of Plant Biology,* **51,** 463–499 (2000).

KaviKishor, P.B., S. Sangam, R.N. Amrutha, P.S. Laxmi, K.R. Naidu, K.R.S.S. Rao, S. Rao, K.J. Reddy. P. Theriappan and N. Sreenivasulu: Regulation of praline biosynthesis, degradation, uptake and transport in higher plants: Its implications in plant growth and abiotic stress tolerance. *Curr. Sci*., **88**, 424-438(2005).

Lutts, S.V. Majerus and J.M. Kinet: Effects of salt stress on growth, mineral nutrition and proline accumulation in relation to osmotic adjustment in rice (*Oryza sativa* L.) cultivars differing in salinity resistance. *Plant Growth Regul.*, **91**, 207- 218 (1996).

Lutts, S., V. Majerus and J.M. Kinet: NaCl effects on proline metabolism in Rice (*Oryza sativa* L.) seedlings. *Physiol. Plant*, **105**, 450–458 (1999).

Madan, S., H.S. Nainawatee, R.K. Jain and J.B. Chowdhury: Proline and proline metabolising enzymes in *in-vitro* selected NaCl-tolerant *Brassica juncea* L. under salt stress. *Ann. Bot.,* **76**, 51–57 (1995).

Mattioni, C., N.G. Lacerenza, A. Troccoli, A.M. De Leonardis and N.Di Fonzo: Water and salt stress-induced alterations in proline metabolism of *Triticum durum* seedlings. *Physiol. Plant*, **101**, 787–792 (1997).

Rhodes, D., P.E. Verslues and R.E. Sharp: Role of amino acids in abiotic stress resistance in plant amino acids (Ed.: B.K. Singh). Mercel Dekker Inc., New York, pp. 319-356 (1999).

Roosens, N.H.C., J.T.T. Thu, H.M. Iskanda and M. Jacobs. Isolation of the Ornithine-δ-aminotransferase cDNA and effect of salt stress on its expression in *Arabidopsis thaliana*. *Plant Physiol,* **117**, 263–271 (1998).

Rout, N.P. and B.P. Shaw: Salinity tolerance in aquatic macrophytes: probable role of proline, the enzymes involved in its synthesis and

C4 type of metabolism. *Plant Sci.,* **136**, 121-130 (1998).

Sudhakar, C., P.S. Reddy and K. Veeranjaneyulu: Effect of salt stress on the enzymes of proline synthesis and oxidation in greengram (*Phaseolus aureus* Roxb.) seedlings. *J. Plant Physiol.,* **141**, 621–623 (1993).

Turan, M.A., H.A.E. Abdelkarim, T. Nilgun and T. Suleyman: Effect of salt stress on growth, stomatal resistance,praline and chlorophyll concentrations on maize plant. *Afr. J. Agril. Res*., **4**, 893-897 (2009).

USDA: World Rice Production, Consumption and stock, *Rice Alamanac*., 3 edn., pp. 59- 235 (2008).

Vogel, R.H. and M.J. Kopac: Some properties of ornithine-α-transaminase from Neurospora. *Biochem. Biphys. Acta,* **37**, 539-540 (1960).

Wang, K., L.Y. Kuanhu, D. Jie., D. Junmei., K. Qingchuan, Y. He and S. Yan: The effect of NaClon proline metabolism in *Saussurea amara* seedlings. *Afri. J. Biotech*., **10**, 2886-2893 (2011).

Yoshiba, Y., T. Kiyosue, K., Nakashima, K,. Yamaguchi-Shinozaki and K. Shinozaki: Regulation of levels of proline as an osmolyte in plants under water stress. *Plant Cell Physiol*., **38**, 1095-1102 (1997).