In silico and expression analysis of Δ¹-pyrroline-5-carboxylate synthetase in rice seedlings under NaCl stress

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Abstract
The synthesis of proline, an osmolyte, is one of the stress response mechanisms of the plants. Glutamate is converted to proline by the activity of the enzyme Δ¹-pyrroline-5-carboxylate synthetase (P5CS). We have analyzed the gene (p5cs) behind this enzyme to study the relationship between the expression of p5cs and the proline content of different rice genotypes. The shoots of the genotypes showing salt tolerance (SR26B and Nonabokhra) revealed a significant positive correlation between the elevated expression of p5cs and proline level, while the roots showed insignificant correlation. Danaguri, a rice landrace, on the contrary, showed a negative correlation between the expression of p5cs and proline in both shoots and roots.

The salt-sensitive rice genotype (IR64) indicated a positive correlation between the expression of p5cs and proline in both shoots and roots. Considering a feedback inhibition of the activity of P5CS by proline, we performed in silico molecular docking experiment. We observed a very close competition between the binding sites of P5CS with glutamate, the substrate and proline, the product.

Keywords Rice, NaCl stress, *p5cs* gene expression, proline, *in silico* docking, glutamate, feedback inhibition.

Introduction

The accumulation of proline subject to abiotic stress like salinity is a general phenomenon in plants. Its diverse role in stress alleviation is also widely worked out[6,13,21]. Despite that, the aspect of proline accumulation is still an enigma, particularly in the genotypes showing sensitivity or tolerance to salt. In plants, proline synthesis mainly follows either of the two paths the glutamate and ornithine[7,8].

The enzyme Δ¹-pyrroline-5-carboxylate synthetase (P5CS) catalyzes the formation of Δ¹-pyrroline-5-carboxylate (P5C)[15], an intermediate in the pathway which is then acted upon by the enzyme Δ¹-pyrroline-5-carboxylate reductase (P5CR) to produce proline[12,32].

In the ornithine pathway, ornithine produces proline by the action of the enzyme ornithine-δ-aminotransferase; it is then transaminated to P5C[8,9]. However, proline accumulation in plants like rice during stress mainly followed the glutamate pathway[13,21].

Δ¹-pyrroline-5-carboxylate synthetase (P5CS) and Δ¹-pyrroline-5-carboxylate reductase (P5CR) are the two final enzymes, which participate in the formation of proline via the glutamate pathway. While P5CS catalyzes the penultimate step in this pathway, P5CR catalyzes the ultimate stage of converting P5C (pyrroline-5-carboxylate) into proline. Surprisingly, enhancing P5CR level in plant cell does not help in accumulating higher amount of proline[21]. Even 200 times increased expression of P5CR led to no noticeable proline increment in transgenic tobacco[32].

On the contrary, increasing the amount of P5CS leads to a higher amount of proline accumulation in a cell[1]. Overexpression of P5CS in transgenic plants increases proline production and osmotolerance[19]. Overexpression of *GmP5CS* increased proline accumulation in *Glycine max*[4]. Hence, P5CS is a crucial enzyme in the synthesis of proline from glutamate.[7,16,19,29,32,33]

Synthesis of proline from glutamate by P5CS is subject to feedback inhibition. The product (proline) and the substrate (glutamate) both compete for the active site of the enzyme P5CS[3,25,26]. In plants growing under stress, the accumulation of proline may be due to the loss of feedback inhibition as a result of a change in conformation of the P5CS protein. The conformational change of the protein and subsequent loss of feedback inhibition were achieved by replacing Phe (129) with Ala in *Vigna aconitifolia*[14]. It has been also seen that expressing P5CS by replacing Phe at 129th position with Ala (P5CSF129A) in transgenic rice[20] and pigeon pea[31] growing under salinity stress helped to accumulate higher amount of proline than salt-stressed non-transgenic plants.

In *Arabidopsis* there are two paralogs (p5cs1 and p5cs2) of the gene p5cs encoding the enzyme P5CS[30]. In rice, the enzyme P5CS is synthesised by two isoforms of the gene *p5cs*, *Osp5cs1* and *Osp5cs2*[37]. Rice is susceptible to salinity. The activity of *Osp5cs* and proline accumulation was more pronounced in a salt-tolerant rice variety under high salinity than that in a salt-sensitive breeding line[17].

Contrary to it, there is opposite report available in barley, where proline accumulation is an indication of salt susceptibility with no role in salinity tolerance whatsoever[5]. Therefore, we can see that though proline accumulation has

been reported as a tolerance response of plants to combat salinity, deviations exist and there lies the enigma.

Here, in this study we have quantified the amount of proline accumulated in the root and shoot of four *indica* rice genotypes differing in salt tolerance ability. They were grown under salinity and also under control conditions. The objective was to observe how salinity stress affected proline accumulation in the salt-tolerant and salt-sensitive rice genotypes. Expression levels of the gene *p5cs* in the stressed and unstressed plants of each rice genotype were measured by qRT-PCR to correlate the level of accumulation of proline in the roots and shoots. In some cases, higher expression of the *p5cs* did not show a corresponding high accumulation of proline. It is, therefore, necessary to determine whether there exists a one to one relationship between increased *p5cs* expression and proline accumulation.

Of many probable reasons, feedback inhibition of P5CS enzyme can be one of the causes behind the inconsistency of proline accumulation under salt stress in different genotypes and tissues. We have used few bioinformatic tools to resolve the issue. After sequencing the partial coding sequences of the *p5cs* from the four rice genotypes, we have docked the respective predicted proteins with glutamate, the ligand. Additionally, considering the competition between glutamate and proline for the binding site in P5CS, the protein structure derived from the common sequence of all the genotypes in study and the full-length predicted sequence of *p5cs* in *Oryza sativa* as obtained from NCBI was docked with both the ligands.

Material and Methods

Rice genotypes: The present study includes four *indica* rice genotypes e.g. IR64, Danaguri, SR26B and Nonabokhra. IR64 is a high yielding yet salt sensitive rice cultivar developed by IRRI and grown worldwide[23]. Danaguri is an aromatic landrace of rice grown in few districts of West Bengal, India. SR26B and Nonabokhra are salt tolerant rice varieties released from IRRI[10]. Seeds of the four rice genotypes were germinated in autoclaved de ionised water. After germination the seeds were placed in 0.5 % (85 mM), 1% (171 mM) NaCl solution and one set of seeds of each genotype was germinated in water as a control. The germinated seedlings were allowed to grow for ten days in this condition. Lengths of root and shoots were measured for ten plants from each set of each genotype chosen at a random.

Quantification of Proline: An equal amount (100 mg) of tissue (root and shoot separately) of each genotype was crushed with 3% (v/v) sulphosalicylic acid. After filtration of the slurry, we added glacial acetic acid (1 ml) and acid ninhydrin (1 ml) to the filtrate followed by thorough mixing in a cyclo mixer and boiling in a water bath. Finally, we separated the proline with toluene (3 ml) using a separating funnel. We recorded the absorbance of the samples at 520 nm using a UV-VIS spectrophotometer. We followed the protocol of Bates et al [2] to quantify the proline content of the samples against a standard curve of pure proline. The experiment was done with three replicas for both shoot and root of each of the rice genotypes grown in control and saline conditions.

Design of primers for the *p5cs*: From the NCBI database, we obtained the sequence of *Oryza sativa* Δ'-pyrroline-5-carboxylate synthetase (*p5cs)* gene (GenBank: XM_015784690). We used the coding region to prepare gene-specific primers - one for cloning and sequencing the gene, the other for quantitative real-time PCR analysis (Table 1). We used the Os_NABP (*Oryza sativa* Nucleic acid- binding-protein) as the constitutive gene [28].

RNA isolation and cDNA preparation: We isolated the total RNA from 100 mg tissue (root and shoot separately) of the four rice genotypes grown in deionised water (control), 0.5% and1% NaCl solution. We used the RNA isolation kit of SIGMA (SpectrumTM) for this procedure. We estimated the concentration of RNA using a Thermo Scientific-make Nanodrop Spectrophotometer. The quality-checking of RNA was done by resolving it on 0.8% (w/v) agarose gel. Using a dedicated kit (Quanti-Tect® QIAGEN) for Reverse Transcription, we followed the protocol of the manufacturer for the synthesis of cDNA. It was used as the template for real-time PCR.

Table 1
List of primers

Name of primers	Primer sequence (Forward, F and Reverse, R) (5′– 3′)	Ta (standardized annealing temperature)	Target amplicon size (bp)
P5CS1	F- CTGTGCGAGCAGGTTAAGGA R- ACAGGTGTGCCGCTATTTGA	60ºc	625
P5CS2	F- TCTGTGCGAGCAGGTTAAGG R- GCCATCAGTCCACTCTGACC	60ºc	195
OsNABP	F- GGAATGTGGACGGTGACACT R-TCAAAATAGAGTCCAGTAGATTTGTCA	60ºc	100

Real-time RT PCR: We performed the reactions using the Maxima SYBR Green/ROX qPCR kit of Thermo Scientific in an Applied Biosystems® 7500 fast real-time PCR machine. Of the total reaction mixture (25µL), the amount of cDNA, the template was 2 µL. We used the constitutive *NABP* gene for normalizing the relative value of expression of the target gene. We followed the formula of Livak and Schmittgen[22] for the calculation of the fold increment of the gene. We represented the data of qRT-PCR as the mean of three replicates with the standard deviation and performed the Student's t-test to analyze the level of significance between the mean values of data of plants grown in control, 0.5% and 1% salt solutions.

Amplification and cloning: The cDNA of shoots and roots of the four rice genotypes was the templates for PCR reactions. Each PCR reaction mixture consisted of 5× NH_4 buffer (2.5 µL), $MgCl_2$ (25 mM, 2.5 µL), dNTP (200 µM, 2.5 µL), Taq polymerase (0.5 U), 100 µM primer (0.5 µL), template (25 ng, 2 µL) and deionised PCR-grade water to make up the volume to 25 µL. We performed the PCR reactions using a 2720 Thermal Cycler apparatus (Applied Biosystems). The cycle of the reaction was as follows: initial denaturation at 94°C (2 min), then 29 cycles each of denaturation at 94°C (1 min), annealing at 60°C (1 min) and1 min extension at 72 °C (1 min). There was a final extension period at 72°C for 7 min. We resolved the amplified products on agarose gels (1.6% w/v, 1× TAE, 7 V/cm). We used the Gel Extraction kit (QIAGEN) for elution and purification of the amplified products.

For cloning the products, we used the TOPO TA cloning Kit (Invitrogen) and transformed those into *E. coli* TOP10 strain. We screened the resulting clones on LB agar plates with kanamycin (150 µg /mL) as the selection antibiotic. Subsequently, we used another kit (Invitrogen) for the isolation of the plasmids from the transformed colonies. It followed restriction digestion of the plasmids with EcoR1 for confirmation of the presence of inserts.

Finally, we did the sequencing of the cloned fragments following the dideoxy chain termination method using universal primers (M13 F and M13 R) with the automated sequencing machine (Applied Biosystems) at the Central Instrumentation Facility of Bose Institute, Kolkata.

Analysis of the nucleotide sequence: We used the obtained sequences of the four rice genotypes separately as the query sequences for homology searching using the NCBI database to look for their percentage of similarity with the predicted sequence present in the NCBI database. Subsequently, we performed the multiple sequence alignment analysis along with the full-length predicted sequence of p5cs gene using the Clustal Omega tool. We recorded the mismatches in the nucleotide sequence among the four genotypes.

***In silico* characterization of P5CS:** The amino acid sequences of the P5CS enzyme of the different rice genotypes were obtained with the help of the Expasy Translate tool. Each amino acid sequence was submitted to the I-Tasser server (https://zhang lab.ccmb.med.umich.edu/ ITASSER/)[36] to obtain the probable secondary and tertiary structures of the enzyme. The ligand binding sites in the enzyme were identified by COACH (https://zhang lab.ccmb.med.umich.edu/COACH/)[34] and the ligand information was obtained from the BioLip database[35].

***In silico* modelling and structural analysis:** We used the SWISS-MODEL[11] package for the generation of the predicted protein models of P5CS for the four rice genotypes. Next, we checked the stereo-chemical stability of the models and obtained the Ramachandran plots for each of the models.

Docking analysis: We uploaded the mol2 files of glutamate and proline separately in the SWISS-Dock server (in the ligand selection tab). We took the help of UCSF Chimera to generate the files of glutamate and proline. After opening the .sdf files (from PubChem database) and the addition of the hydrogen atoms, we saved those in the mol2 format. Of the various numbers of possible chains provided by SWISS-Dock, the best possible ligand - protein interaction was chosen by docking the ligand to the binding residues present in the active site. These residues were obtained from *in silico* molecular docking in ITasser server. The two ligands were docked with the same protein chain using Tools/ Structure Analysis/ View Dock/ Choose chain (to be done multiple times for the correct one)/ Cluster pdb/ pdb 4 5 or 6.

Statistical analysis: We performed the Paired *t*-test of the relevant results using GraphPad QuickCalcs and correlation analysis using Minitab Statistical software.

Results and Discussion

Effect of NaCl on growth, *p5cs* expression and proline content

In salt sensitive genotype (IR64): The length of shoots reduced significantly with increasing NaCl concentration concerning the control (Table 2), the decline being 41% and 72% in 0.5% and 1.0% NaCl respectively (Fig. 1a). The length of roots also showed significant decline, reduction being 38% and 42% in 0.5% and 1.0% NaCl respectively (Table 2, Fig. 1a). The level of proline accumulation was significantly higher both in the shoots and roots of the seedlings grown in NaCl (0.5% and 1.0%) with respect to the control seedlings (Table 3, Fig. 1a). The expression of *p5cs* initially showed an insignificant decline (0.5% NaCl) but it increased significantly later (1.0% NaCl) both in the shoots and roots in comparison to the control set of plants (Table 4, Fig. 1a).

In landrace (Danaguri): The length of shoots reduced significantly with increasing NaCl concentration concerning the control (Table 2), the decline being 56% and 81% in 0.5% and 1.0% NaCl respectively (Fig. 1a). The length of roots also showed significant decline, reduction being 66%

and 78% in 0.5% and 1.0% NaCl respectively (Table 2, Fig. 1a). Proline content revealed a steady significant increment in the shoots in comparison to the control set in both the NaCl concentrations. The accumulation of proline was low in the roots, though it showed a significant increase in 0.5% NaCl over the control, the proline content came to the level of the roots of the control plants in 1.0% NaCl (Table 3, Fig. 1a). The expression of *p5cs* showed an insignificant decline in the shoots in both the NaCl concentrations with respect to the control. On the contrary, the expression of *p5cs* declined significantly in both the NaCl concentrations in roots in comparison to the control set of plants (Table 4, Fig. 1a).

In salt tolerant genotypes (SR26B and Nonabokhra): In both the genotypes, the length of shoots showed significant decline with increased NaCl concentration in comparison to their respective control sets. The length of roots showed a significant increment in 0.5% NaCl but it declined in 1.0% NaCl in comparison to the control set in both the genotypes (Table 2, Fig. 1b). Proline concentration increased significantly with increasing NaCl concentrations in the shoots and roots of both the genotypes as compared with the respective control sets (Table 3, Fig. 1b).

The expression of *p5cs* showed an ascending trend in the shoots of both SR26B and Nonabokhra. The trend was reverse in case of the roots where the *p5cs* expression declined with increase in both the NaCl concentrations in both the genotypes (Table 4, Fig. 1b).

In the present study we tried to correlate proline synthesis and salt stress with *p5cs*, the gene behind the crucial enzyme in the glutamate pathway of proline biosynthesis, operational in rice during salt stress.

Table 2
Length (mm)* of shoots and roots in four rice genotypes under NaCl stress

NaCl conc. (%)	IR64		Danaguri		SR26B		Nonabokhra	
	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
0	51.6±3.12	41.6±3.88	49.78±1.9	42.78±0.09	65.6±2.8	27.4±3.2	66.4±4.11	30.4±4.47
0.5	30.2±2.26	25.8±2.2	21.9±1.77	14.6±0.02	49.6±1.63	41.6±4.47	53.8±1.77	43.2±4.88
1.0	14.4±1.72	24±0.89	9.6±1.41	9.3±0.41	11±2.28	22.6±4.42	14±1.89	24.4±5.09

*(mean ± SE, n=5)

Table 3
Proline content (µg/µl)* of shoots and roots in four rice genotypes under NaCl stress

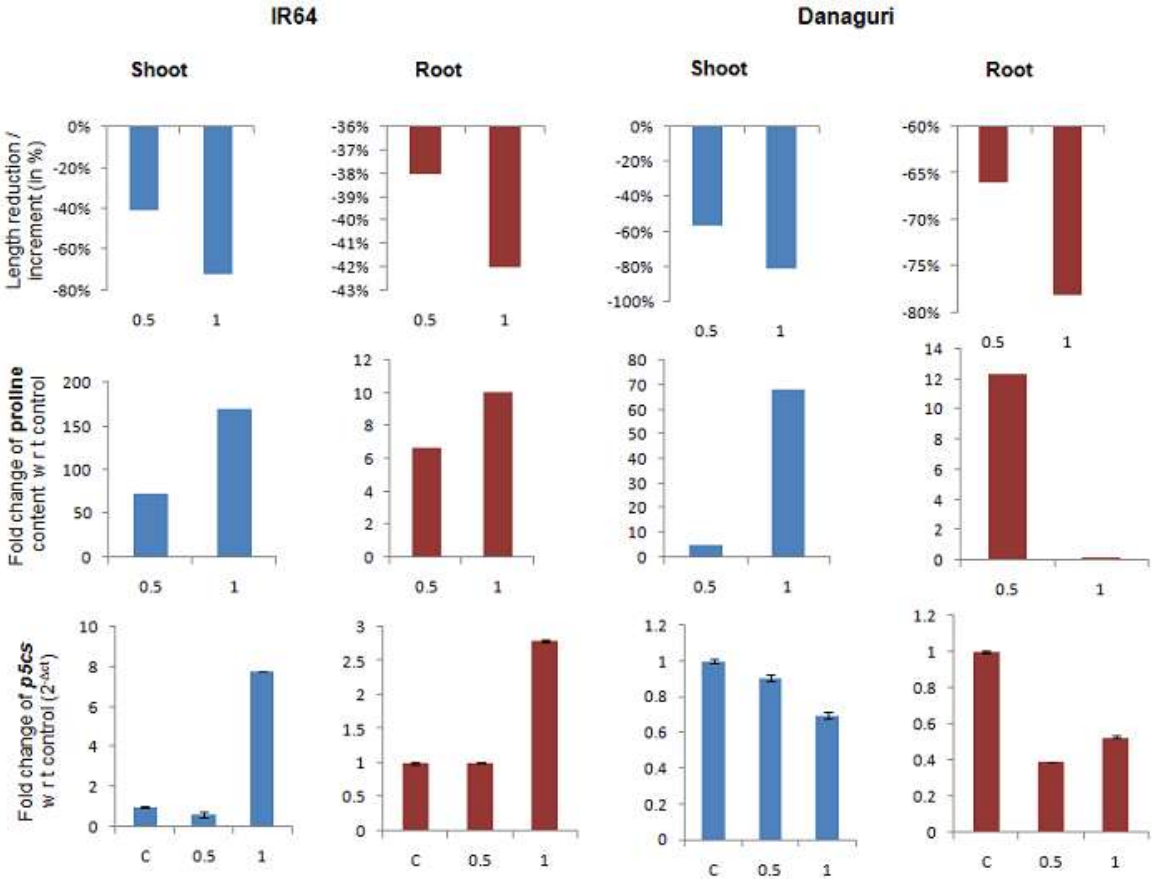
NaCl conc. (%)	IR64		Danaguri		SR26B		Nonabokhra	
	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
0	15.92±1.32	3.23±0.33	57.66±3.69	10.82±2.13	5.34 ± 0.58	2.756± 0.23	24.8±1.24	18.6±0.76
0.5	88.22±11.30	9.88±1.36	62.79±5.09	23.13±2.23	206.56±6.50	11.16±2.32	34.98±1.41	16.02±0.47
1.0	185.44±24.25	13.3±3.07	125.87±2.4	9.35±2.10	229.28±8.08	11.49±3.80	153.47±14.61	23.67±4.69

*(mean ± SE, n=5)

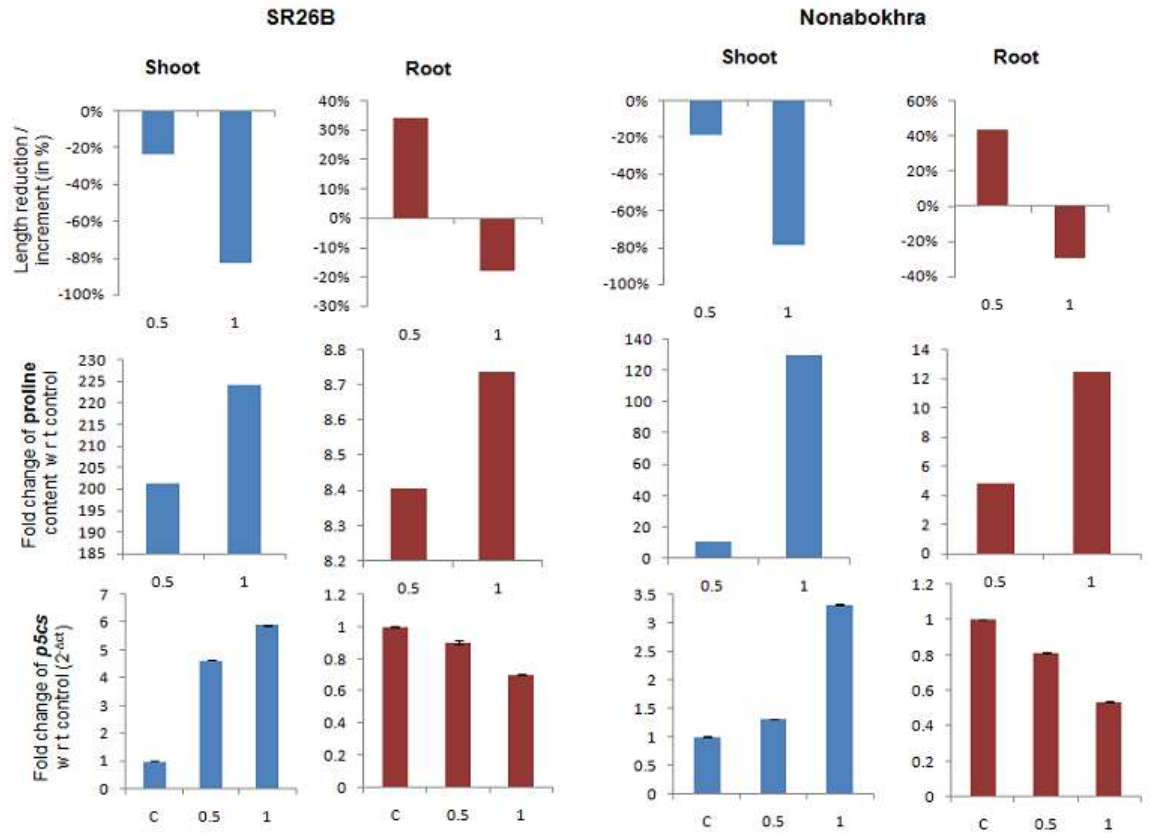
Table 4
Levels of significance (P values from Student's t- test) between the differences of mean value in four rice genotypes under NaCl stress. A: difference between control set and seedlings grown in 0.5% NaCl, B: difference between control set and seedlings grown in 1.0% NaCl. ^{NS} – Non Significant difference, Relative decrease ↓, Relative increment ↑

Genotype	Length				P5cs expression*				Proline Accumulation			
	Shoot		Root		Shoot		Root		Shoot		Root	
	A	B	A	B	A	B	A	B	A	B	A	B
IR64	↓P=0.00	↓P=0.00	↓P=0.0	↓P=0.02	↓P=0.35 ^{NS}	↑P=0.00	↓P=0.95 ^{NS}	↑P=0.02	↑P=0.01	↑P=0.01	↑P=0.01	↑P=0.04
Danaguri	↓P=0.00	↓P=0.00	↓P=0.00	↓P=0.00	↓P=0.72 ^{NS}	↓P=0.11 ^{NS}	↓P=0.00	↓P=0.01	↑P=0.03	↑P=0.00	↑P=0.00	P=0.48 ^{NS}
SR26B	↓P=0.00	↓P=0.00	↑P=0.05	↓P=0.02	↑P=0.00	↑P=0.03	↓P=0.50 ^{NS}	↓P=0.02	↑P=0.04	↑P=0.00	↑P=0.04	↑P=0.02
Nonabokhra	↓P=0.04	↓P=0.00	↑P=0.00	↓P=0.02	↑P=0.05	↑P=0.00	↓P=0.06	↓P=0.00	↑P=0.00	↑P=0.01	↑P=0.01	↑P=0.02

*calculated from the mean $2^{-\Delta ct}$ values



(a)



(b)

Figure 1: Percent reduction/increment of length, fold change of proline accumulation and *p5cs* gene expression of shoots and roots in the seedlings grown in 0.5 and 1.0% NaCl respect to control in four rice genotypes: (a) IR64 and Danaguri, (b) SR26B and Nonabokhra.

Table 5
Positive and negative correlation (Pearson correlation coefficient, r values) between proline accumulation and *p5cs* expression of shoots and roots in four rice genotypes with increased NaCl concentration (0, 0.5 and 1.0%)

Genotype	Shoot	Root
IR64	0.833	0.762
Danaguri	-0.976	-0.677
SR26B	0.987	-0.587
Nonabokhra	0.999	-1

Table 6
The results of the *in silico* docking experiment: The amino acids of P5CS in the binding site with its ligand (glutamate)

Genotype	Amino acids in the binding site and their position	Predicted distance (Å) between ligand and the amino acid
IR64	Asn (67), Asp (70), Trp (87), Asn (89)*****	4.143
Danaguri	Asn (81), Asp (84)*****, Asp (102), Asn (103)	1.802
SR26B	Asn (67), Asp (70), Asp (88), Asn (89)*****	2.788
Nonabokhra	Asn (67), Asp (70), Asp (88), Asn (89)*****	2.438

***** Amino acid binding with the ligand

Since P5CS is the rate-limiting enzyme, we can expect a direct relationship between *p5cs* expression and proline accumulation. However, we found few deviations in this hypothesis, particularly in genotypes differing in salt tolerance and their organs like shoots and roots.

We observed a significant positive correlation between *p5cs* expression and proline accumulation in the shoots of Nonabokhra and SR26B. In the shoots of these salt-tolerant genotypes, both *p5cs* expression and proline concentrations increased than the control set of plants in both the NaCl concentrations. In contrary, in the roots of these two genotypes, the correlation between *p5cs* expression and proline accumulation was negative; the proline levels increased, yet the *p5cs* expression decreased with the increase in NaCl concentration (Table 6).

In the case of Danaguri, the rice landrace, the correlation between the *p5cs* expression and proline accumulation was negative, irrespective of shoots and roots. Contrary to it, there was a positive correlation between the *p5cs* expression and proline accumulation in both shoots and roots in IR64, the salt-sensitive rice genotype (Table 6).

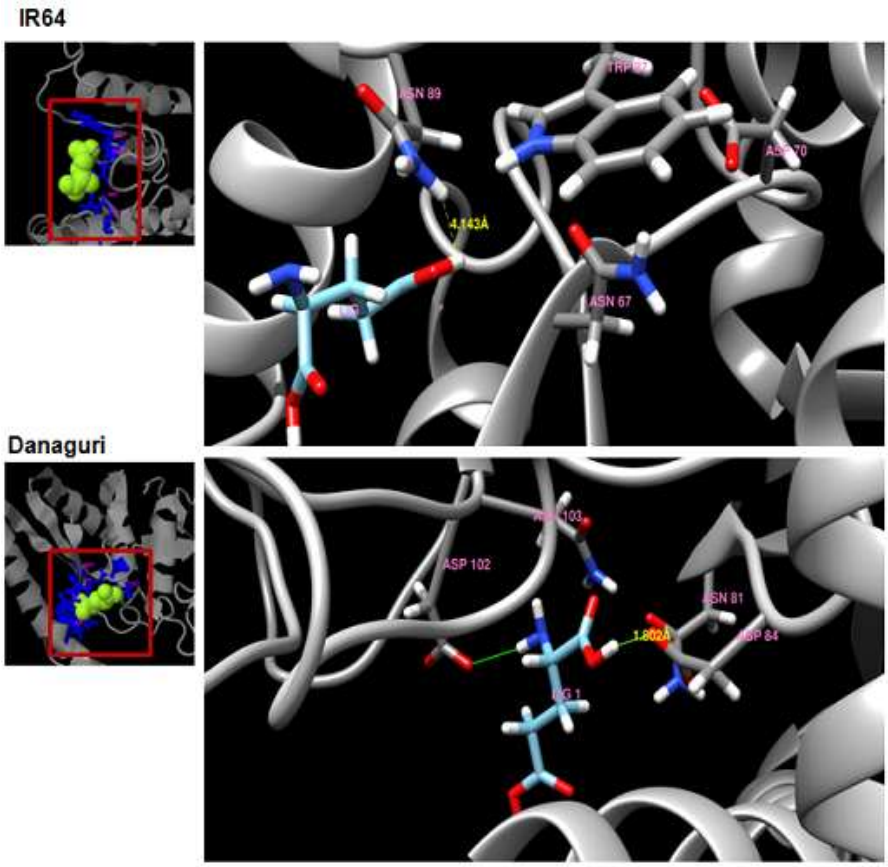
Therefore, in the salt-sensitive rice genotype, a direct relationship is tenable between *p5cs* expression and a higher level of proline accumulation under saline condition. A similar relationship exists even in the shoots of the salt-tolerant genotypes. However, the negative correlation between *p5cs* expression and proline accumulation in the roots of the salt-tolerant genotypes possibly indicates a different mechanism operational in roots. NaCl-induced proline accumulation was many-fold in the shoots in all the genotypes compared to that in the roots. It again substantiates the possibility of the existence of different mechanisms of salt tolerance in shoots and roots.

The mechanisms that a plant employs to resist the ill-effects of salinity can be broadly divided into three phases - osmotic phase, ionic phase and tissue tolerance phase[27]. The tissue tolerance phase is characterised by the synthesis of osmolytes like proline, glycine-betain, sucrose etc.[27] This stage is functional in the shoots under high saline conditions[27].

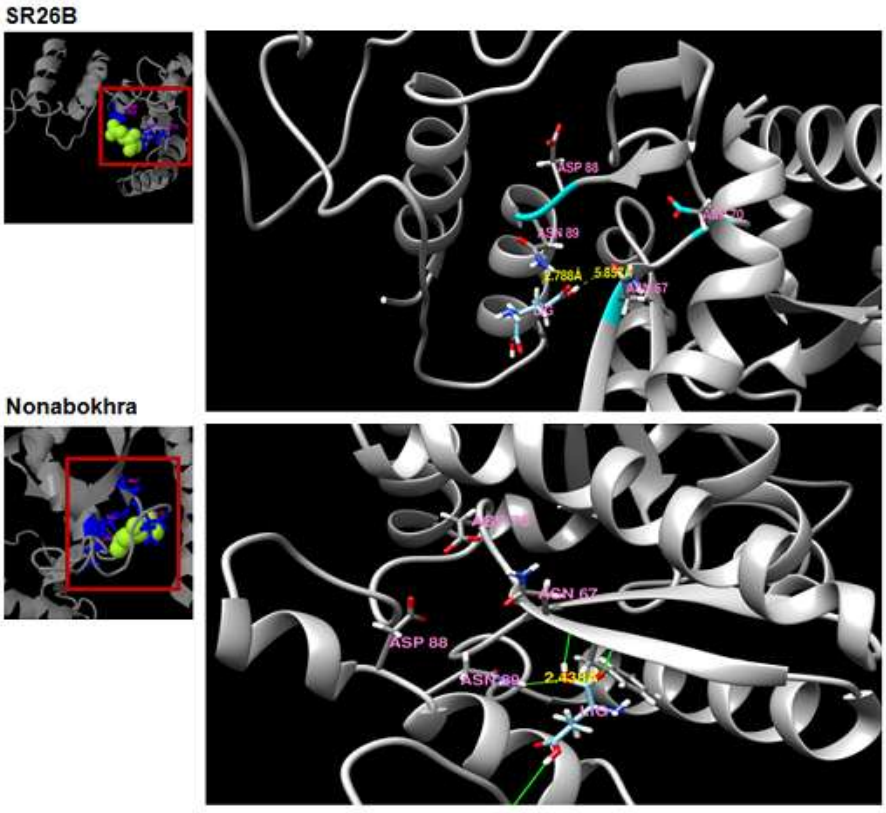
The reason that proline accumulated in lesser amounts in the roots than the shoots in all the rice genotypes under study may be because the tissue tolerance phase is functional in the shoots and not in the roots. The osmotic phase is marked by the rapid transmission of signals from the roots to the shoots, the organ for future resource allocation during the transition from vegetative to reproductive stage.

As a result, the synthesis of osmolytes increases much more in the shoots than the roots. The degree of osmotic tolerance depends on various factors such as the initial perception of signal, rate of transmission of the signal for long-distance signalling [24]. Therefore, it is plausible that in salt-tolerant genotypes, this osmotic tolerance mechanism is highly efficient, resulting in a higher activity of *p5cs* in the shoots and a consequent higher proline accumulation whereas, in the salt-sensitive genotypes, the signal perception and consequent transmission are weak; the phenomenon of *p5cs* expression and proline accumulation in different organs is probably not well-sequestered between roots and shoots [18].

Of the four genotypes under study, the findings of Danaguri seem to be the most difficult to explain. The proline accumulation in both roots and shoots under salt stress is a generalist event in this genotype too. It probably points out the omnipresent osmoregulatory role of proline subject to salt stress irrespective of genotype.



(a)



(b)

Figure 2: Molecular docking of P5CS with its ligand, glutamate in four rice genotypes: (a) IR64 and Danaguri, (b) SR26B and Nonabokhra. Enlarged representations of the marked portions are visualized by UCSF Chimera1.14 software.

However, the *p5cs* expression never increased significantly in either roots or shoots, making the explanation inconclusive. We can only presume that its genetic makeup is different since it is a landrace and yet to subject to human selection.

***In silico* studies on P5CS:** Glutamate is the substrate (ligand) of the protein P5CS in all the four rice genotypes as obtained from molecular docking. The amino acid residues of the ligand-binding site of P5CS are near conserved in the four genotypes. It was identical in SR26B and Nonabokhra, the two salt tolerant genotypes with the sequential arrangement of Asn, Asp, Asp and Asn at the 67, 70, 88 and 89th position. In IR64, the salt sensitive genotype, the Asp residue of 88th position was replaced by Trp at 87th position. An arrangement of the amino acids similar to the salt tolerant genotypes was also observed in Danaguri, the rice landrace. However, their relative position was different (at the 81, 84, 102 and 103rd position) (Table 5, Fig. 2a, b).

Of the four amino acids at the ligand-binding site, the last one i.e. Asn at the 89th position showed the minimal distance with glutamate, the ligand in three genotypes viz. IR64, SR26B and Nonabokhra. In Danaguri, however, Asp at 84th position was closest to the ligand (Table 5, Fig. 2a, b). The distance between the ligand (glutamate) and the definite amino acid in the binding site to which it binds was obtained to be < 5 Å in all the four genotypes (Table 5).

We anticipated that looking for a direct relationship between the expression of *p5cs* and accumulation of proline cutting across, all the genotypes might not be successful; the reason being in addition to transcriptional control, there is a potent feedback inhibition of the activity of P5CS by proline, the product emanating from the reaction[38]. We took the help of few bioinformatics tools to study the P5CS protein of the four genotypes. In spite of the highly conserved amino acids present in the ligand-binding site of P5CS in the four genotypes, we observed a difference in the substrate-binding site in case of IR64, the salt sensitive genotype. Only in this case one tryptophan residue is present in place of aspartate present in the other three rice genotypes.

Furthermore, molecular docking of IR64-P5CS revealed a very close competition between the binding sites of P5CS with glutamate, the substrate (1.848Å) and proline, the product (1.773Å) (Figure 3). Can we presume that in IR64, a salt-sensitive genotype proline has out-competed the substrate earlier limiting its production? On the other hand, in the salt tolerant genotypes and the landrace proline took relatively more time to inhibit the substrate allowing more proline to accumulate to alleviate the salt stress response.

Conclusion

We found a distinct difference in proline accumulation and salinity tolerance level in the rice genotypes. We conclude that a differential expression of p5cs exists between salt-tolerant and salt-sensitive rice genotypes. The salt-tolerant genotypes employ different mechanisms besides proline synthesis to combat salinity more efficiently. Concerning the feedback inhibition of proline by glutamate, the substrate in the active site of enzyme P5CS, our *in silico* study provided direct evidence of very close distance between proline, glutamate and the active site of P5CS in rice.

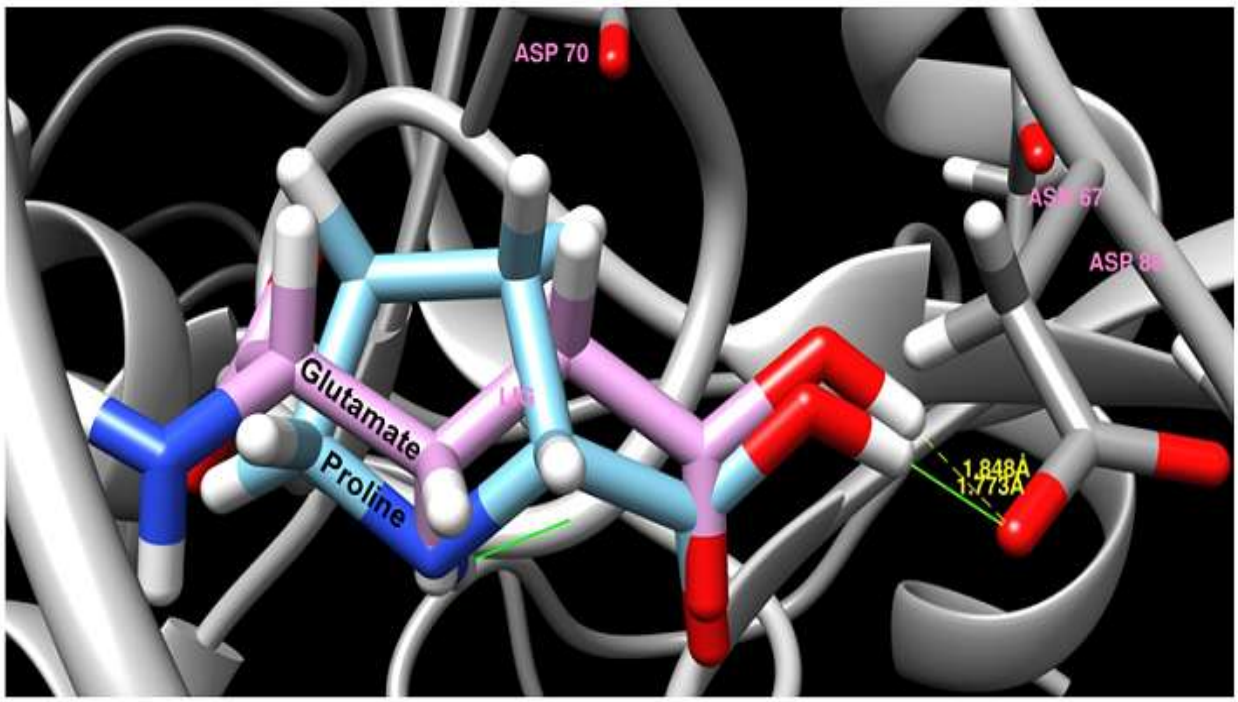


Figure 3: Enlarged portion of the molecular docking of P5CS with two ligands: glutamate (the substrate) and proline (the product) considering the full-length predicted sequence of *p5cs* in *Oryza sativa*.

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