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Silicon Mediated Alleviation of Salinity Stress Regulated by Silicon Transporter Genes (*Lsi1* and *Lsi2*) in Indica Rice

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HIGHLIGHTS

- Silicon alleviates salinity in rice by mitigating the effect of ionic, osmotic and oxidative stress.
- Both Si transporters (*Lsi1*and *Lsi2*) were up-regulated under salinity stress.
- *Lsi1* transporter is concerned with maintaining osmotic balance, while *Lsi2* transporter is responsible for excess Na^+ ion exclusion from cell during high salinity.
- *Lsi2* expression was found to be highly variable among rice genotypes.

Abstract: Silicon accumulation is known to improve tolerance of plants under both biotic and abiotic stress. Salinity stress is an inevitable crisis causing wide spread damage to rice leading to food insecurity. The influence of Si (1mM) on two rice cultivars cv. Ghanteswari (high accumulator) and cv.Badami (low accumulator) which differs in Si uptake potential under saline (10ds/m EC) and non- saline conditions were studied in nutrient culture. The Si transporter genes were isolated and characterized to determine their function in salinity tolerance. Under stress, there was an increase in Si accumulation, Na^+/K^+ ratio, electrolyte leakage, lipid peroxidation and antioxidant activities. On addition of silicon, the K^+ uptake increased, membrane damage reduced and osmolytes balance improve under salinity. But, the level of resurgence was varied in both cultivars, due to their differential Si-accumulation. Molecular characterizations of *Lsi1* protein revealed its involvement in the movement of ion and water and therefore prevent osmotic stress. The *Lsi2* is responsible for removal of Na^+, reducing salt toxicity. Silicon accumulation is responsible for maintenance of cell water status, osmotic balance and Na^+ ion exclusion during high salinity. The variable relative expression of *Lsi2* provides a possible explanation for differential genotypic uptake of silicon.

Keywords: differential Si-uptake; indicarice; *Lsi1* and *Lsi2* gene expression; Silicon; salt tolerance; qRT PCR.

INTRODUCTION

Salinity is a pronounced complex ecological factor affecting agriculture by altering growth and development of plants. Around 20% of total cultivated land and 33% of irrigated agricultural lands are affected by high salinity [1]. In India, 6.74 million ha area is adversely affected by salinity resulting in a decline in production and productivity [2]. Salinity occurs as a result of nutritional toxicity, water stress and accumulation of excess sodium ions (Na^+) in the plant species [3]. The salinity also hampers photosynthesis/carbohydrate metabolism which causes an increase in the generation of reactive oxygen species (ROS), and finally results in oxidative stress in plants [4,5]. The essential staple food of Asia, 'Rice', is highly sensitive to salinity. The rice yields decrease up to 12% per unit increase above 3.0 dS/m in electrical conductivity (EC) [6]. For dealing with the problem of salinity and to ensure food security through agriculture, it is essential to identify the physiological and molecular responses for salt resistance and to evolve strategies that could guarantee crop survival and productivity in an unsafe environment. Silicon (Si) is a known element which improves performance of different plant species and providing both abiotic and biotic resistance [7-12]. Silicon accumulation was also shown to enhance yield and productivity in most of the terrestrial and aquatic plants, but the rate of accumulation varies greatly among and within species. Silicon prevents structural and functional deterioration of cell membranes under environmental stress, and also involved in the thermal stability of cell membranes [13]. Si increase stress tolerance and decrease membrane damage in tomato (*Solanum lycopersicum*) and spinach (*Spinacia oleracea*) [14]. In drought stress rice, Si (1.5 mM) causes reduces leaf tissue concentrations of potassium, sodium, calcium, magnesium, and iron, but increased chlorophyll concentration [15]. Further, silicon application prevents reduction of photosynthetic rate, transpiration rate, stomatal conductance of stress-induced plants [16]. In rice, highest silicon accumulation up to the level of 10% of shoot dry weight was reported by various authors, so the trait could be exploited for building better salt tolerant genotypes [7,17]. In Japonica rice, two silicon transporters were reported to be associated with the uptake of silicon from the soil solution to the apoplast, i.e. *Lsi1* and *Lsi2,* respectively [18]. Based on beneficial effect of silicon in rice, the present study focused on evaluating the change occurring in salinity tolerance indices with respect to the differential Si accumulation in rice cultivar subjected to salt stress and determine the mechanism involved in Si-induced salinity tolerance.

MATERIALS AND METHODS

Plant materials and nutrient culture

Two indica rice genotypes, Ghanteswari (high Si accumulator) and Badami (low Si accumulator) were collected from Rice Research Station of OUAT, Odisha. These genotypes having differential Si-uptake potential were screened for their salt tolerance level at the most salt-sensitive stage of the crop, i.e. seedling stage. The salt-tolerant variety 'Lunishree' and the salt-sensitive 'IR29' were used as standard check cultivars in salt screening. Seeds of all four cultivars were surface sterilized with 0.1% (w/v) mercury chloride for two minutes and 0.5% bavistin (w/v) for fifteen minutes and were allowed to germinate in dark at 28℃ for five days. Seedlings of equal size were selected and transplanted on nylon net frame fitted in plastic bowls containing Yoshida Nutrient Solution (YNS) and were exposed to light (3000 lux) for 12 h photoperiod with 25℃ day/night temperature [19]. After two weeks, the planted nylon net frames of bowls were finally fitted into the styrofoam boards. The styrofoam boards were allowed to float on plastic pots filled with 3 litre of Yoshida culture solution (pH-5.0±0.5). These pots were then placed in a glasshouse maintained at 28±2℃ with 50% relative humidity. The culture solution was changed at four- day intervals and EC was maintained. The pH and reduced water level were also maintained.

Treatment condition

Sodium chloride (NaCl) and diatomaceous earth (SiO_2) were directly added to the nutrient solution after four weeks of sowing. The salinization was done for a week in Yoshida nutrient solution (YNS) to obtain electrical conductivity (EC) of 10ds/m (~100mM), while the non- saline serving as control showed EC of 1ds/m. Thus, the experiment was designed in completely randomized design (CRD) as control without Si and NaCl (only YNS)-T_1 and three combination treatments: Si (1.0mM)-T_2, NaCl (10ds/m EC)-T_3 and Si (1.0mM) + NaCl (10ds/m)-T_4. The young expanded leaves were harvested after 7 days of saline treatment and stored at -20℃ prior to analysis. Measurements on sodium and potassium content, electrolyte leakage percentage (ELP), lipid peroxidation (LPO) level, activities of antioxidant enzymes, proline content, and level of carbohydrates were analyzed.

Silicon accumulation in shoots and roots

The shoots/roots were dried at 70 ℃ for 5 days and ground with liquid nitrogen into a fine powder. Aminomolybdate method was used to determine the Si content in rice [20]. The ground samples of leaves (0.1 g) were digested with 3mL of 50% NaOH in a volumetric flask. About 1mL of digested sample solution was transferred to a 50mL polyethylene tube. Further, 30mL of 20% acetic acid and 10mL ammonium molybdate (54 g/L, pH 7.0) were also added. The solution was shaken thoroughly and kept for 5min and then added 5mL of 20% tartaric acid and 1mL reducing solution**.** The volume was made up to 50mL with 20% acetic acid. The absorbance was taken at 650 nm using UV/Vis-Spectrophotometer (LAMBDA 365, Perkin Elmer) after 30 min. The amount of silicon was calculated with standard calibration curve.

Determination of sodium and potassium

Sodium and potassium content in leaves was determined [21]. About 10 mg dried leaves of each sample was cut into 1cm size pieces, placed in respective test tubes containing 20 mL distilled deionized water, and heated 1 hour in a boiling water bath. The tubes were then autoclaved at 121 °C for 20 minutes and cooled. The samples were diluted to 10 times. The elemental analysis was made by atomic absorption spectrophotometer (iCE™ 3300 AAS).

Electrolyte leakage potential

Electrolyte leakage (EL) potential was determined to assess membrane permeability [21]. Hundred miligram fresh rice leaves were cut into 0.5 cm pieces and placed in test tubes. Ten ml distilled deionized water was added to the test tubes and placed in a water bath with constant temperature of 32 °C. After 2 hours, the initial electrical conductivity of the samples (EC_1) was measured. The tubes were later autoclaved at 121 °C for 20 minutes and cooled to 25 °C. Finally electrical conductivity of each sample (EC_2) was measured to expressed electrolyte leakage potential (EL) as $EL=EC_1/EC_2\times100$.

Estimation of protein

Four mL protein extract was prepared by homogenizing 0.5 g of leaf tissue in 50mM phosphate buffer (pH 7.8) containing 1mM EDTA, 2% PVP (w/v) and 0.1% triton X-100.The homogenate was centrifuged at 10,000 rpm for 30min at 4˚C and the supernatant was used for biochemical assays. An aliquot of the extract was used to determine the total protein content in the samples utilizing bovine serum albumin as the standard [22]. Protein (mg/g fresh weight) was calculated using the linear equation: $y = 0.0024x + 0.013$.

Determination of lipid peroxidation (LP)

LP was determined by measuring the concentration of the MDA (Malondialdehyde) content in leaf [23].The reaction mixture consists of a total volume of 4ml containing 1mLof sample extract, 3mLof 2% (w/v) TBA (2-thiobarbituric acid) dissolved in 20% trichloroacetic acid (TCA). The mixture was heated at 95˚C for 30 minutes and then quickly cooled on an ice bath. Then centrifuged at 12,000 rpm for 10 min and the absorbance of the supernatant were read at 532 nm. A correction for the non-specific turbidity was taken at 600nm and subtracted from the absorbance at 532nm.The LP were expressed as nmol MDA g^{-1} fresh weight basis.

Enzymatic study

All antioxidant enzyme assays were studied by using UVis-spectrophotometer (LAMBDA 65, Perkin Elmer) with a total reaction mixture solution of 3 mL, including enzyme extract. All enzymes activity was expressed as unit per min per mg of protein.

Superoxide dismutase (SOD, EC 1.15.1.1)

SOD activity was determined by photochemical reduction of nitro blue tetrazolium (NBT) at 560 nm [24]. The SOD activity was determined by adding 100µL of the enzymatic extract to a solution containing 55µM NBT, 1µM riboflavin, 9.9mM methionine, 2M EDTA and 50 mM phosphate buffer (pH-7.8). The reaction mixture without leaf extract was considered as control and kept in light, while blank including enzyme extract was kept in the dark. One unit of the enzyme activity can be defined as the amount of enzyme required to cause 50% inhibition in the rate of nitro blue tetrazolium.

Catalase (CAT, EC 1.11.1.6)

CAT activity was determined by the oxidation of H_2O_2 [25]. The reaction mixture consists of 50mM phosphate buffer (pH 7.0) and 10mM H_2O_2 solution. The reaction was initiated by the addition of 100µL of crude enzyme extract and the activity was determined by measuring the initial rate of disappearance of H_2O_2 at 240 nm (E = 39.4mM^{-1} cm^{-1}) for 180 seconds.

Guaiacol peroxidase (POX, EC.1.11.1.7)

POXactivity was measured using a modified guaiacol oxidation method [26]. The reaction mixture contained 50mM (pH-7.0) phosphate buffer, 20mM guaiacol (2-ethoxyphenol), 0.042% H_2O_2 and 100µL of enzyme extract. The increase in absorbance as a result of oxidation of guaiacol was measured at 470 nm (E = 26.6mM^{-1} cm^{-1}) for 180 seconds.

Ascorbate peroxidase (APX, EC 1.11.1.11)

APX activity was determined by oxidation of ascorbate [27]. The reaction mixture, with a total volume of 3 mL consisted of 25 mM sodium phosphate buffer (pH-7.0),1mM EDTA, 5mM ascorbate, 0.1mM H_2O_2, and 100 µL of the enzyme extract. The oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm (E = 2.8 mM^{-1} cm^{-1}) for 180 seconds.

Genomic DNA isolation and primer design

All available nucleotide sequences of silicon transporter genes (*Lsi1* and *Lsi2*) were searched and retrieved from NCBI (www.ncbi.nlm.nih.gov) and used for primer designing with Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi). Genomic DNA was extracted from two rice cultivars i.e. Badami and Ghanteswari using the modified C-TAB method The PCR amplification consisted of 35 cycles with 59 ℃ and 52 ℃ annealing temperatures for *Lsi1* and *Lsi2*, respectively. The single amplicon obtained for each gene was eluted using PCR product purification Kit (Geneaid). Elutes obtained were sequenced with modified Sanger method [ABI 3730XL] at AgriGenome Labs Pvt Ltd, Kochi, Kerala, India.

Sequencing of Si transporter genes

The sequences acquired were subjected to BLAST (Basic Local Alignment Search Tool) for homology search. The NCBI ORF finder and Conserve Domain search tool were employed for protein sequence characterization. Multiple sequence alignments of both genes with their respective homologous sequence were performed using COBALT (Constraint-based Multiple Alignment Tool) with amino acids sequences as input, to identify the conserve domains [28]. Motif Scan (https://myhits.isb-sib.ch/cgi-bin/motif_scan) was also used for finding all known motifs that occur in the sequence. The secondary structures were also predicted for both Si transporters by homology modeling through Structural Analysis in Swiss model, Expasy [29]. All amplicons were subjected to Multiple Sequence Alignment (MSA) using muscle programme (www.ebi.ac.uk/Tools/msa/muscle/) by keeping parameters as default. These sequences were finally subjected to MEGA-6 (Molecular Evolutionary Genetic Analysis) for evolutionary history analysis and tree inferred was assessed by 1,000 replicates bootstrap trial using Neighbor-Joining method [30].

RNA isolation and cDNA synthesis

RNA was isolated from 0.1 g of the fresh roots of 5-week-old rice seedlings using Qiagen's RNeasy Plant Extraction mini kit. The RNA quality was checked using 0.5mL cuvette in a UV-spectrophotometer (LAMBDA 365, PerkinElmer) at 260/280 nm. Transcripts showing 260/280 ratios of 1.9 to 2.1 were chosen for first-strand cDNA synthesis. Consequently, sequence-specific first-strandcDNA was synthesized using Verso cDNA Synthesis kit (Thermo Fisher Scientific, Germany) in a 20 µL reaction mixture with thermocycler (BIORAD, USA) following the manufacturers' instructions and finally stored at −20 °C until used.

Relative quantification with qRT PCR

The relative expression level of *Lsi1* and *Lsi2* in each treatment was compared to control and measured by quantitative RT-PCR. q-Reverse Transcriptase PCR was performed using 48-well Applied Biosystems® StepOne™ Real-Time PCR System (Applied Biosystems, CA, USA) using SYBR® Premix Ex Taq™. The total reaction volume of 20µL contained 1µL of 1:3 diluted cDNA, along with 100 nM of each gene-specific

primers and 10µL of SYBR Premix Ex Taq (Takara Bio). Biological triplicates were taken for both genes specific and reference primer in the qRT-PCR analysis to threshold cycle (C_t) values. The primer sequences used were: *Lsi1*, 5'-CAAACTCCAGGGCGAACTAC-3' (forward) and 5'-TCATGAACACCAGCAGGAAC-3' (reverse); 5'-GCTGTTCCTCAAGAGCTTCG-3'(forward) and 5'-AGACGAGCAGCGAGTAGGAC-3' (reverse); Actin (endogenous control), 5'-GACTCTGGTGATGGTGTCAGC-3' (forward) and 5'-GGCTGGAAGAGGACCTCAGG-3' (reverse).

Statistical Analysis

The experiment was set up in completely randomized design (CRD) with factorial arrangement. All experimental data were means of three replicates. ANOVA (analysis of variance) was performed on the data with the preloaded software of data analysis in MS Excel, and significant differences among treatments were separated by the Fischer's least significant difference (LSD) test at a 0.05 probability level. All graphs were presented with standard deviation as error bars.

RESULTS

Silicon mitigating salinity stress in Indica rice

Silicon supplementation enhanced salinity tolerance and promoted growth of rice seedlings. Levels of different biostress markers such as Na^+, K^+, antioxidant enzymes, lipid oxidation related to salt stress were determined to obtain a clear understanding of the degree of reduction in salinity damage caused by silicon accumulation. The mitigation effect of silicon was predictable after 7 days of salinity treatment at 10dS/m [21]. It was found that the cultivars also displayed the genotypic difference in Si accumulation. After screening, two indica cultivars as cv.Ghanteswari (3.79mg Si/g plant dry weight, high accumulator) and cv.Badami (1.67mg Si/g, low accumulator) with differential genotypic Si-accumulation ability were selected for further salinity trial [31].

Silicon deposition

On addition of Silicon, 'Ghanteswari' showed the high Si accumulation in shoot of 2.37mg/g dry weight, while 'Badami' showed lower Si-accumulation with 1.25mg/g dry weight as expected under non-salinized conditions (Figure 1A). On the other hand in salinized medium, both 'Ghanteswari' and 'Badami' showed further higher Si accumulation of 2.97mg/g and 2.01mg/g plant dry weight, respectively. The salinity check genotypes 'IR-29' and 'Lunishree' seemed to be low silicon accumulators based on the data obtained. The silicon concentration in root was found to be distinctly higher than that in the shoot (Figure1B).

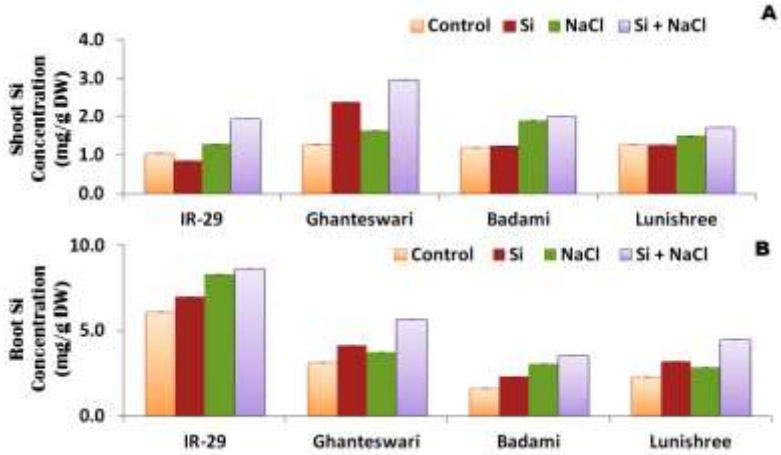

Figure 1. Silicon content estimated from (A) shoots (LSD=0.34) and (B) roots (LSD=0.63) of genotypes in four different treatments expressed as mg/g dry weight of rice.

Ion accumulation

The salt-sensitive check 'IR-29' (69.08mg/kg) showed pronounced Na^+ ion accumulation, followed by 'Ghanteswari' (58.36mg/kg) and 'Badami' (44.48mg/kg) under saline conditions. Low Na^+ deposition is a sign of salinity tolerance as observed in 'Lunishree' (4.21mg/kg). The genotype 'Ghanteswari' showed a lower reduction (48.43mg/kg) than 'Badami' (40.17mg/kg). The K^+ uptake decreased with high salinity and thus the

Na^+/K^+ ratio increases in all cultivars. Si supplementation increases the K^+ uptake, preventing nutrient imbalance and reduces Na^+/K^+ ratio (Figure 2A).

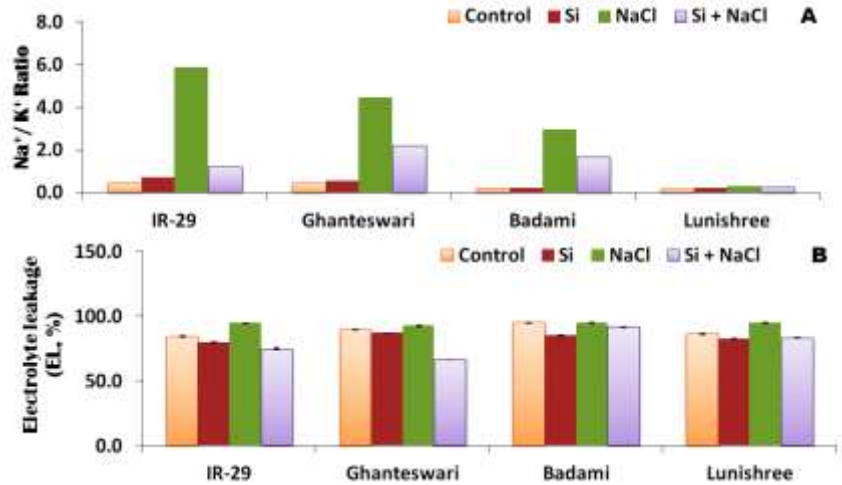

Figure 2. Effect of Si supplementation on (A) ions accumulation (Na^+/ K^+ ratio) (LSD=19.26) and (B) electrolyte leakage potential (EL, expressed in %) (LSD=8.29)

Analysis of membrane stability indices

The addition of silicon in saline solution significantly reduced EL levels in all genotypes, especially in 'Ghanteswari' from 92.92% to 66.99% and in 'Badami' from 95.34% to 91.93% (Figure 2B). The LP decreases in all genotypes with the addition of Si under nutrient medium (Figure 3A), but the salt tolerant genotype Lunishree doesn't show much change in LP even under high salinity, i.e. from 12.01nmole/g FW in control to 13.83nmole/g FW 10ds/m under saline conditions.

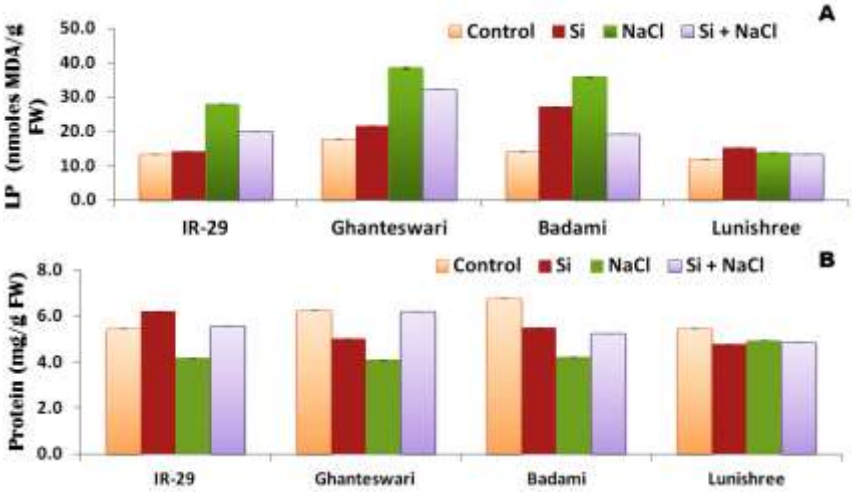

Figure 3. Effect of Si supplementation in rice under saline and non saline culture on (A) lipid peroxidation (LP, nmole MDA/g fresh weight tissue) (LSD=7.79) and (B) Soluble protein (mg/g fresh weight tissue of plant) (p-Value=0.07).

Soluble protein and antioxidant metabolism

Salinity, in general, reduces the soluble protein content of all rice species tested, but the reduction was not significant. The augmentation in protein content was positively related to the amount of Si accumulated, i.e. 'Ghanteswari' showed a clearly higher increase in protein content than 'Badami' after addition of Si in salinized medium (Figure 3B).We focused on the Si induced variations in the antioxidant potential of enzymes, i.e. SOD, CAT, POX and APX under salt stress. Except for salt-sensitive 'IR29' cultivar, all genotypes showed enhanced SOD activity under saline conditions. With the addition of Si, the SOD activity was reduced in 'IR29' and increased in Si accumulating 'Ghanteswari' (Figure 4A). In case of CAT, salinity leads to enhanced enzyme activity (Figure 4B). Silicon treatment under saline and non-saline conditions further reduced the enhanced level of CAT. In POX, salinity increases POX activity in all genotypes. But, Si inclusion again increases POX activity significantly in 'Ghanteswari' (Figure 4D), while reducing its activity in

salt tolerant genotype under stress. However in APX, the enzyme activity reduces in response to salt stress, but the inclusion of Si results into enhanced activity during stress period (Figure 4C).

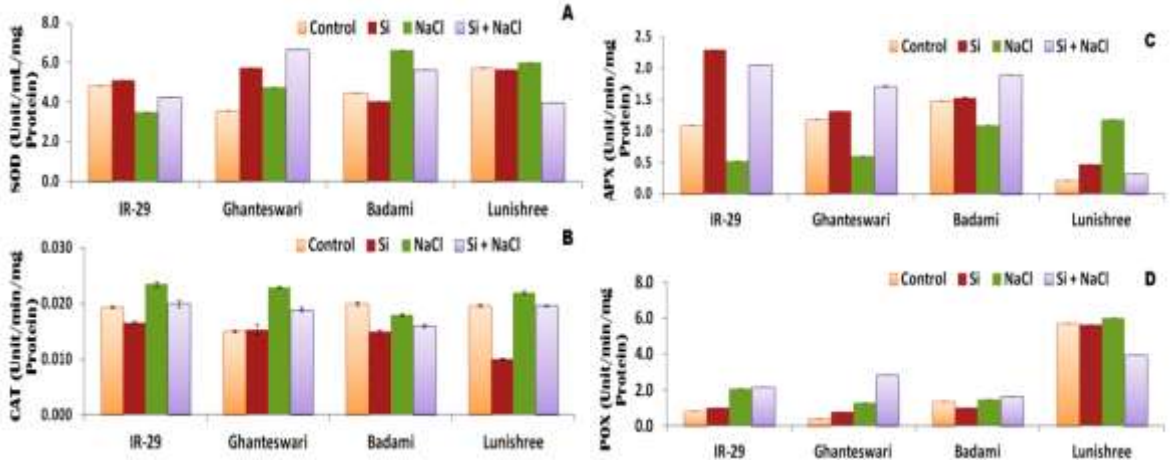

Figure 4. Moderation in behavior of antioxidant enzymes after Si supplementation under salinity stress: (A) SOD (NBT reduced/min/mg protein), (B)CAT (µmole of H_2O_2 decomposed/min/mg protein) (LSD=0.01), (D) GPX (µmole of Guaiacol decomposed/min/mg protein) (LSD=1.25), (C) APX (µmole of Ascorbate decomposed/min/mg protein).

Isolation and characterization of Si transporter genes (Lsi1 and Lsi2)

The *Lsi1* and *Lsi2* genes produced a single sharp and bright fragment of approximately 700 bps and 1400 bps, respectively in both the genotypes. The PCR products obtained as a single band were directly purified and loaded in the gel again to confirm their concentration for quality sequencing (Figure 5). The BLAST search conducted for *Lsi1* of 'Badami' and 'Ghanteswari' detected high homology of 97% and 96%, respectively with *Oryza sativa* Indica Group (cv.Shuhui 498) chromosome 2 sequence (CP018158.1). Also, it showed 95% homology with both the coding sequences of *Oryza sativa* Indica Group cv.Dular NOD26-like major intrinsic protein (MIP) (GU980690.1) and *Oryza sativa* Japonica Group *Lsi1* mRNA for NOD26-like MIP (AB222272.1), when matched with cv.Badami. While, the sequence of *Lsi1* of cv.Ghanteswari showed 1% less, i.e. 94% homology with both the above stated coding sequences. Similarly, BLAST search for the query sequence of a *Lsi2* transporter gene in cvs. Badami and Ghanteswari showed homology of 99% and 100%, respectively with the *Oryza sativa* Indica Group (cv.Shuhui 498) chromosome 3 sequence (CP018158.1). *Lsi2* query sequence of both cultivars showed highest homology (99%) with both the coding sequences of *Oryza sativa* Japonica Group *Lsi2* for transmembrane protein, complete (AB222273.1) and *Oryza sativa* Indica Group B2 isolate of low silicon transporter 2 (*Lsi2*), (HG531963.1).

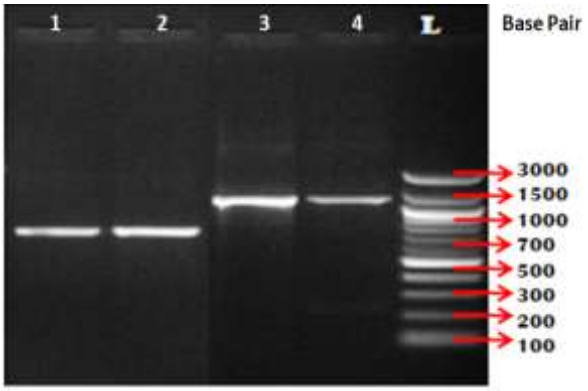

Figure 5. Amplified product of *Lsi1* gene (~700bp) and *Lsi2* gene (~1400bp) of two rice cultivars cv.Badami (1,3) and cv.Ghanteswari (2,4) with ladder (L) in agarose gel

Molecular characterization of transporter genes

ORF (Open Reading Frame) finder encoded the presence of 117aas in genotype Badami and 102aas in genotype Ghanteswari of *Lsi1* gene fragment. Another, search tool for Conserve Domains identification of NCBI used for predicting the function of the *Lsi1* protein, showed that the protein is associated with the Major Intrinsic Protein (MIP) super family. They are water channel proteins generally having six transmembrane helices structure commonly called as aquaporins (AQPs). The protein sequence of *Lsi1* gene, thus identified

was subjected to MSA through COBALT protein tool and compared the *Lsi1* sequences of cvs. Ghanteswari and Badami with the sequences of *Oryza indica* and japonica group obtained during homology search in BLAST (Figure 6A-B). The Motif scan search carried out to identify any possible motifs, could not obtain a definite match. The 3D protein model proposed by Swiss model in cv.Badami (Figure 7A) showed resemblance with the monomeric form of 'large-conductance mechano-sensitive channel' with 17.78% sequence identity and 0.38 sequence coverage. Similarly, Swiss model for cv.Ghanteswari protein (Figure 7B) predicted monomeric 'Hemoglobin-like protein' structure with 13.46% sequence identity and 0.51 sequence coverage. The Swiss model showed the ligand of protein to be "protoporphyrin IX containing FE" or "phosphate ions" in predicted structure, which were engaged in transport functions. The aligned sequences were further used for preparing phylogenetic tree to identify the evolutionary ties between the cultivars. In case of *Lsi2* gene fragment of cv.Badami and cv.Ghanteswari, the ORF finder translated both the nucleic acid into a single continuous amino acid sequence of 208aas. The NCBI Conserve Domains hit showed high resemblance with 'Anion permease (ArsB/NhaD) superfamily'. A typical anion permease contains 8-13 transmembrane helices and can function as an independent chemiosmotic transporter. COBALT protein tool was again used for Lsi2 protein MSA. The *Lsi2* conserve protein when submitted to Motif scan tool provided positive hits, and the motif formed was of 'citrate transporters, (pfamls:CiMHS)'. Also, the 3D homology modeling was executed for each cultivar and monomeric structure proposed for both the cultivar proteins showed high resemblance to 'Transporter, NadC family' (Figure 7C-D), with 19.82% sequence identity and 0.53 sequence coverage. The obtained model also projected the ligands for the protein structure to be "sodium ions" and "sugar (B-Nonylglucoside)". A cluster analysis was performed between aligned nucleotide sequences (through Muscle) of *Lsi1* and *Lsi2* genes of cv.Ghanteswari and cv.Badami with other consensus sequences of Si transporter genes reported in different crops (from NCBI). The reported *Lsi1* gene sequence of *Sorghum bicolor* (KF793921.1) showed 97.37% similarity with gene sequence of cv.Ghanteswari and 92.39% with cv.Badami. The transporter gene sequence of *Zea mays* (DQ524811.1) showed 86.67% and 88.70% similarity with cv.Ghanteswari and cv.Badami, respectively. The consensus sequence of MIP (Major Intrinsic Protein) in *Triticum aestivum* (AK457911.1) and *Hordeum vulgare* (AB540229.1) showed 95.31% similarity with *Lsi1* gene sequence of both cultivars. Similarly in case of *Lsi2* gene, the percentage similarity between consensus sequence of *Sorghum bicolor*, *Zea mays, Triticum aestivum*, *Hordeum vulgare,* etc. ranges from 80-86% (Figure 8A-B).

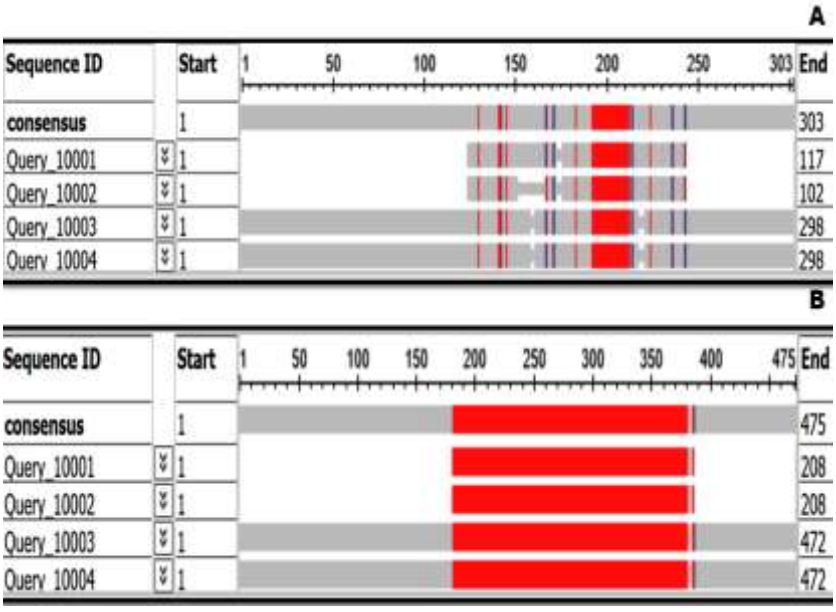

Figure 6. Graphical representation of conserved domain of (A) *Lsi1* protein MSA with four query sequences, (B) *Lsi2* protein MSA with four query sequences

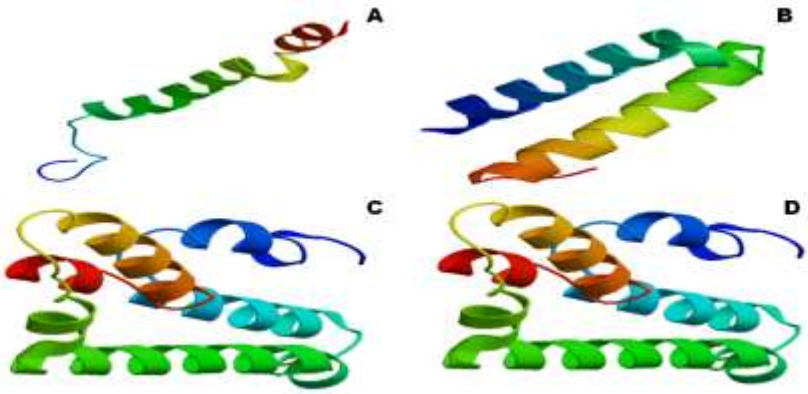

Figure 7. Homology model of predicted tertiary structure of *Lsi1* and *Lsi2* genes performed using SWISS-MODEL based on crystallographic data deposited on the Swissprot: (A) *Lsi1* of cv.Badami, (B) *Lsi1* of cv.Ghanteswari, (C) *Lsi2* cv. Badami, (D) *Lsi2* of cv.Ghanteswari.

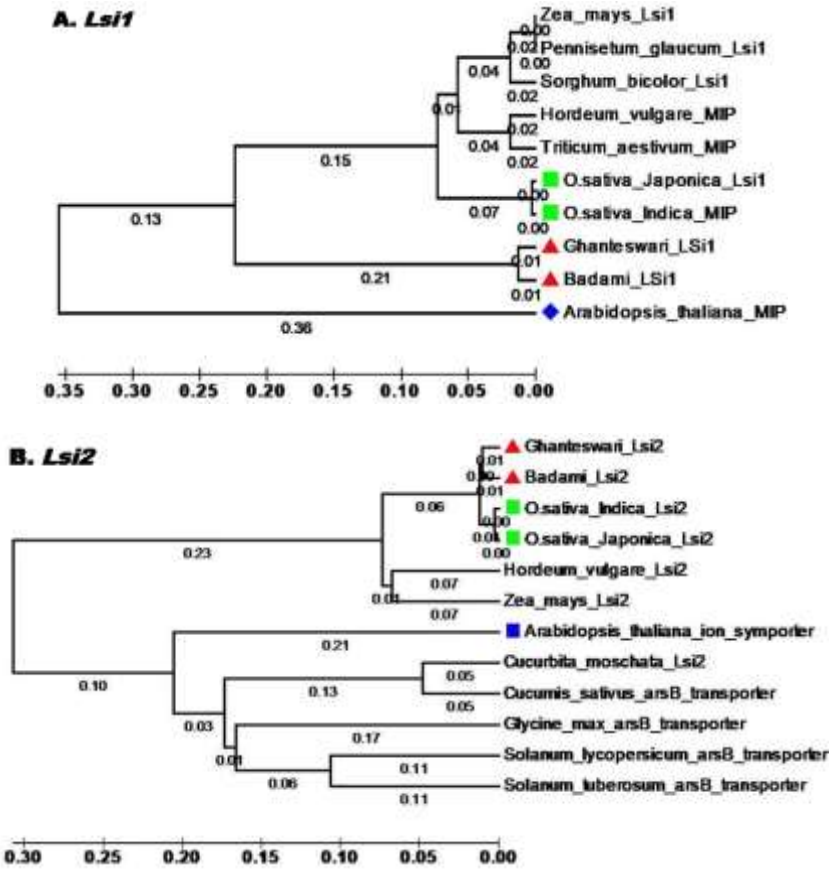

Figure 8. Cluster analysis of silicon transporter genes (A) *Lsi1* and (B) *Lsi2* of rice with silicon transporter gene reported in other species.

Relative expression of Lsi1 and Lsi2 genes under salinity

Both the genes showed higher relative expression under salinity signifying enhanced need of Si uptake during salinity. With the addition of Si in saline treatment, the rate of expression of both genes was reduced. The up and down regulation of genes can be clearly determined by fold change graph (Figure 9A-B). The relative expression level of *Lsi1* under salinity was up-regulated by 690% in cv. Ghanteswari and 507% in cv.Badami. Further, with the supplementation of Si, the relative up-regulation in the rice was reduced to 47% and 17%, respectively. On the other hand, the relative expression *Lsi2* was up-regulated by 424% in cv.Ghanteswari and 138% in cv.Badami. With Si supplementation, the rate of up-regulation is again reduced in to126% and 116%, respectively.

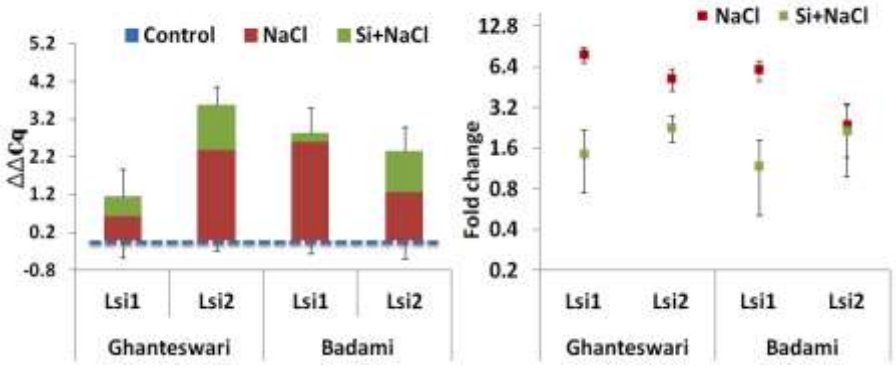

Figure 9. Relative expression patterns of *Lsi1* and *Lsi2* genes under salinity stress conditions.
(A) Expressing positive $\Delta\Delta C_q$ values as up-regulation of genes for each salinity treatment relative to control, (B) Representing fold change increase in expression relative to control.

DISCUSSION

Silicon accumulation in both shoots and roots of all cultivars were determined separately. Both shoot and root tissues showed a significant difference in accumulation of silicon in the four different treatments as well as among the genotypes [31]. The physio-biochemical parameters were compared in relation to the rate of Si accretion to establish a connection between the decline in salinity damage to Si accumulated. The Si concentration estimated was notably higher in roots than the shoot Si content. Similar findings were demonstrated that the Si concentrations in both shoot and root tissues were significantly different in spinach and tomato [14]. However, in case of tomato they reported that Si accumulation higher in shoots than the roots. Sodium (Na^+) and potassium (K^+) contents were determined to get an insight into the mechanism of action of Si against salinity stress. The present results indicate that there was significant accumulation of sodium ion in the shoot of rice seedlings under saline-treatment than in the non-saline conditions. Besides Na^+, K^+ ion which highly influences salinity stress, intake is considerably lowered during the uptake of Na^+ ions. Application of Si, enhances the K^+ ion level under saline conditions. Hence, with the addition of Si the Na^+/K^+ ratio decreases, this is positive index towards salt tolerance. Such ionic co-relationship of Si was also reported in spinach, tomato and maize [14,32].

The electrolyte leakage potential (EL) and lipid peroxidation (LP) of the cell were considered as indicators of cellular damage. EL monitored the extent of membrane damage and integrity in a cell, whereas LP assessed the oxidative burst induced by salinity. Salinity stress trends to increase cell damage by enhancing EL. Therefore, it was concluded that high Si accumulator cultivar showed a high reduction in EL than the lower accumulator. Under stress and non-stress conditions, all genotypes showed a drop in cell membrane damage. Similar results were demonstrated in cucumber and chili under salinity stress with addition of Si application [23,33]. Salinity induces overproduction of ROS (Reactive Oxygen Species) which causes lipid, protein and DNA damage. So, mitigating LP and regulating antioxidant activity are essential. The imbalance in EL leads to enhanced LP, which was determined by measuring the MDA (Malondialdehyde) content an end product of lipid peroxidation. Addition of Si in saline culture significantly decreases LP in all cultivars. Our findings are corroborate with the findings of other researchers in tomato and cucumber under salt stress [23,34-35]. The results also showed a drop in protein level, after addition of NaCl in the hydroponic culture. But the addition of Si in the saline culture boosted the protein content, and such result has also been reported in cucumber and tomato [23,34]. The protein content of plant was also a marker of the normal enzymatic functioning of the plant cell. The reduction in protein content showed hindrance in enzymatic physiological processes of a cell. There was a considerable variation in the protective mechanisms against reactive oxygen species in different plants; it was not easy to establish the general validity of this phenomenon in salt tolerance [36]. The SOD and POX activity were variable responses among cultivars [21]. However, there was a positive association of SOD, POX, CAT, and APX with the differential salt tolerance ability of two rice cultivars [37]. The salt-sensitive genotypes showed a decrease in SOD activity, while the salt tolerant genotypes showed an increased level of SOD under stress [23]. The enzymes CAT, POX, and APX are involved in toxic H_2O_2 decomposition in the cell, which is increased as a result of stress in plant species. Such findings were in agreement with the findings of maize and cucumber [32,35]. Thus, Si inclusion during salt stress was useful for preventing oxidative stress due to accumulating H_2O_2 in the cell periphery.

The gene-specific primers were designed from the conserve domain of close homologs and synthesized for the isolation of partial fragments of Si transporter genes. The sequence obtained was subjected to BLAST for retrieving homology sequences from the Database NCBI. The *Lsi1* gene showed a high homology of with accession no. CP018158.1 suggested the presence of *Lsi1* gene loci on chromosome-2 of rice. Again, the *Lsi2* gene showed a high homology of with accession CP018158.1, providing evidence of the existence of *Lsi2* gene region on chromosome-3 of rice. The ORF (Open Reading Frame) finder of NCBI translated the nucleic acid sequences in respective functional amino acid (aa) sequences. The ORF of *Lsi1* was found to be truncated in case of both the cultivars. MSA was done through COBALT for both the transporter genes that used protein conserve domain database and protein motif information for alignment of sequences. The red highlighted region in MSA of Lsi1 and Lsi2 protein represents the conserved protein regions of gene. The graphical representation of Lsi1 depicted truncated protein consensus sequences, which suggested the possibility of presence of introns in between the consensus domains. Unlike Lsi1, the Lsi2 protein sequences of the cultivars studied and *Oryza* group depicted continuous consensus domain region in all submitted query sequences. The cluster analysis depicted the level of homogeneity between the consensus gene sequences of silicon transporter genes and the evolution trend in gene across different genera. The phylogenetic tree showed higher evolution in the *Lsi1* gene sequence among different species rather than *Lsi2* gene sequences.

The Lsi1 protein act as a member of MIP superfamily so functions as membrane channels that are involved in the selective transport of water, small molecules and ions in between and out of the cells. Hence, we can assume that the Lsi1 protein was also involved in the selective transport of water and ions from the cell surroundings, i.e. enhancing the water status and uptake of ions such as K^+ in the cell. Also, we could propose that the increment observed above in K^+ content with Si addition under salinity in all four genotypes is because of *Lsi1* transporter gene. As a result, the osmotic stress caused during salinity in rice is reduced. So, in rice ionic and water status remain maintained even in high saline condition, which is considered as a prime reason for Si induced salinity mitigation. The Lsi2 protein was also found to be very important in salinity tolerance, as they belonged to Anion permease superfamily. So, they were primarily involved in translocation of sodium, arsenate, sulphate and other organic anions across biological membranes. It was conclude that *Lsi2* proteins were involved in the movement of Na^+ and other toxic ions such as arsenic out of the cell. Throughout the salinity, imbalance of nutrients was observed with high Na^+ uptake causing ionic stress to the plant cell. Therefore, during salinity *Lsi2* transporter induces Na^+ exclusion from the stress cells, while enhancing Si accumulation in the shoot. This explains the result of a reduction in Na^+ content in all genotypes by Si supplementation under salinity, which is the second major key point for Si induced alleviation of salt stress.

The relative expression of *Lsi1* and *Lsi2* genes as compared to the controls were studied in each cultivar under salinity to understand the outcome of Si application and the genotypic difference in Si accumulation. The quantitative-Reverse Transcription PCR (qRT–PCR) was employed to recognize the change in transcriptional response of the genes to the stress conditions by relative quantification of m-RNA in different samples. The data obtained were analyzed in a most common model of $2^{-\Delta\Delta C_t}$ method [38]. The real time results were further standardized for each of the biological replicates. The relative expression results of salinity stress as compared to the control revealed up-regulation of both the *Lsi1* and *Lsi2* genes in all treatments, as the values of $\Delta\Delta C_t$ for all the treatments were positive. The $\log_2$ fold change relative to control was studied for a better understanding of expression level. There was high genotypic variation in expression level of *Lsi2* gene than *Lsi1*. The *Lsi2* gene was involved in translocation of Si from root cell to apoplast [18]. Thus, dissimilarity in the *Lsi2* expression level could be a possible explanation for the genotypic differential Si-uptake and accumulation in cultivars. The variation in *Lsi2* transcripts may be due to variation in promoter region or post- translation modifications. Such results of *Lsi2* variability in transcription were also in accordance with the phylogenetic variations obtained between cultivars.

CONCLUSION

There are three main salinity tolerance mechanisms, which include ion exclusion, tissue tolerance, and shoot ion-independent tolerance [39]. The findings showed that Si could alleviate salinity by ion exclusion and also adopt shoot ion - independent tolerance (enhancing photosynthesis, maintaining osmotic homeostasis). Silicon supplementation regulates the physiological and biochemical mechanisms of rice such as biomass, photosynthesis, nutrient imbalance, cellular oxidation and osmolyte balance resulting into increased growth and yield under salinity. However, the mitigation response level varies with the genetic nature of cultivars; as the study showed that the low Si accumulator cultivar ‘Badami’ couldn’t show efficient

salinity mitigation in comparison to high accumulator 'Ghanteswari'. In conclusion, the variation in Si-uptake potential and accumulation are an essential criterion to which decides the level of mitigation of salinity damage by silicon. So, only Si application as a fertilizer was not sufficient to obtain the complete benefits of the quasi-essential element. Hence, manipulations in Si-transporters (especially *Lsi2*) through breeding programmes and cisgenic introduction will provide a genetic improvement in rice.

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