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MOLECULAR CHARACTERIZATION OF RICE ANTIOXIDATIVE ISOENZYMES AND THEIR TISSUE SPECIFIC RESPONSE TO EXOGENOUS SPERMIDINE DURING SALINITY IN TWO CULTIVARS

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ABSTRACT: Mitigating effect of exogenous spermidine (Spd) treatment on the reactive oxygen species (ROS) scavenging system in response to salinity has been investigated in root and shoot tissues of two Indica rice varieties, namely IR 36 (salt sensitive) and Nonabokra (salt-tolerant). Rice seedlings (5-Day old) were grown in 150mM sodium chloride (NaCl) in presence or absence of 1 mM Spd for 16 h and stress-evoked change in physiological parameters, accumulation of H_2O_2 content, gene expression and biochemical activities of three antioxidative enzymes, such as, catalase, ascorbate peroxidase and superoxide dismutase were determined. Plant growth retardation in terms of chlorophyll degradation, reduction in carotenoid content and enhanced anthocyanin content during salinity exposure were rescued due to external Spd supplementation. Moreover, exogenous application of Spd differentially influenced the antioxidative enzymes systems in root and shoot tissues of two rice cultivars of different sensitivity towards salt stress. The gene expression analyses of different antioxidative isoenzymes revealed that different isoforms of a particular antioxidative enzyme contributed unequally to make a direct correlation with their cumulative enzymes activities. In addition to that some *in silico* structural attributes including 3D protein structure, active site and ligand site prediction have added additional information on our existing knowledge that will be beneficial for the future investigation on the specific potential roles of different isoforms of those anti-oxygenic enzymes in stress management.

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1. INTRODUCTION

Soil salinity is a major environmental factor that shows detrimental effects on agricultural yield mainly due to decreased growth rates, reproductive development and reduced tillering of crop plants eventually resulting in loss of productivity worldwide [1]. The redox balance is interrupted with advent of salt stress, lead to the change in equilibrium between Reactive oxygen species (ROS) such as superoxide ($O^{2-\cdot}$), hydrogen peroxide (H_2O_2), singlet oxygen ($_1O^2$), hydroxyl radicals ($OH^{-\cdot}$) and antioxidants (ROS scavenging compounds). As a result of which plant cells rapidly accumulate reactive oxygen species (ROS), a phenomenon, widely known as the "oxidative burst" that has an important role in inducing signaling events and is dependent on enzymes located in several subcellular compartments [2]. It is essential to keep a tight rein on the ROS production in a coordinated manner via many enzymatic and non-enzymatic pathways as excess production of ROS accelerates uncontrolled oxidation resulting in lipid peroxidation in cellular membranes, DNA damage, protein denaturation, carbohydrate oxidation, pigment breakdown, an impairment of enzymatic activity and ultimately leads to cell death [3,4]. Superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) are vital enzymes that participate in "keeping active oxygen under control". The SODs dismutates O^{2-}. to H_2O_2 and O_2, CAT and APX detoxify H_2O_2 into H_2O and O_2 although the later has the a higher affinity for H_2O_2 than the former that uses ascorbate as substrate [2].

Polyamines (PA), including putrescine (Put, a diamine), spermidine (Spd, a triamine) and spermine (Spm, a tetramine), are small polycationic aliphatic amines that have shown omnipresent distribution throughout the plant kingdom. Positive charges of PAs at physiological pH levels enable them to bind negatively charged molecules like nucleic acids, proteins, and phospholipids [5]. Plant accrued endogenous PA concentration by modulating PA metabolic pathway that contribute in many cellular processes, such as, ROS and ion homeostasis, osmoregulation, hormone production, thus helps them to conquer salt stress induced damage [2]. Many previous works have recommended the exogenous treatment of PAs as more suitable and efficient mind-set to alleviate salt tolerance of plants that ultimately lead to the improvement of crop productivity under high salinity [5,6]. The intricate associations of PA, ROS and antioxidants are known to stimulate several opposing physiological effects during stress which has got establishment from numerous studies on the relationship between PAs and the ROS, especially in plants under stress [6,7]. Rice (*Oryza sativa* L.) is the world's most important staple food being used as fundamental food resource for large part of the world's human population, especially in Asia. Due to having their substantial genetic variability, rice plants exhibits differential striving response to the most common abiotic stress factor, salt. The indica varieties Nonabokra and IR-36 are classified as highly salt-tolerant (STC) and salt-sensitive (SSC) cultivar respectively [8]. To our knowledge, many previous reports successfully ascertained the ameliorative effect of PAs that act as functional elicitors for modulation of stress response in plants under salt

stress [9,10]. In our present investigation we have focused on the antioxidative defence system regulation under immediate onset of salt stress in presence or absence of exogenous Spd. There are many isoenzymes in rice antioxidative systems that have shown difference in their expression pattern in response to environmental constraint conditions [11]. Keeping in mind the above observations we have performed the structural characterizations including 3D protein structures, active site and ligand binding site prediction along with their functional analyses at the transcript level during salt stress in presence and absence of exogenous Spd. These studies will fill the lacuna in our existing knowledge about the potentiality of different isoforms as stress manager during salt stress response.

2. MATERIALSAND METHODS

2.1 Sequence and database search for rice antioxidant isoenzymes

We have retrieved the genomic, coding sequences (CDSs) and protein sequences of each of the splice variant of each of the isoenzymatic genes of antioxidative enzymes from Oryza sativa genome database available in TIGR Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/analyses search locus.shtml). The GenBank accession numbers and the length of nucleotide and amino acid sequences are given in **table 1.**

Table 1 The GenBank accession number and the length of nucleotide and amino acid sequences of each of rice antioxidant isoenzymes genes.

Gene Name	Accession Number	Genomic length (bp)	CDS length (bp)	Protein length (aa)
CAT A	LOC_Os02g02400	2795	1479	492
CAT B	LOC_Os06g51150	4171	1479	492
CAT C	LOC_Os03g03910	3310	1479	492
Cyt APX	LOC_Os03g17690	3421	753	250
Thyl APX	LOC_Os02g34810	3820	1437	478
Strml APX	LOC_Os04g35520	4363	1080	359
Mn-SOD	LOC_Os05g25850	4786	696	231
Cu/Zn-SOD	LOC_Os08g44770	3226	636	211
Fe-SOD	LOC_Os06g05110	4147	768	255

2.2 Plant materials and growth conditions

Two varieties of *Oryza sativa* L. seeds, salt sensitive (IR 36) and salt tolerant (Nonabokra), were obtained from Rice Research Station (Chinsurah, West Bengal, India) and Central Soil Salinity Research Institute (Canning, West Bengal, India) respectively. Surface sterilization of the seeds was done with 0.1% (w/v) $HgCl_2$ for 5 min. After extensive washing seeds were imbibed in deionized water for overnight and allowed to germinate over water-soaked sterile gauge in Petridishes at 37°C in dark for 2 days. The germinated seedlings were grown in Murashige and Skoog (MS) complete

media for another 5 days at 37°C under 16 h light and 8 h dark photoperiodic cycle with 50% relative humidity in a plant growth chamber (G1000, BLUESTAR). For our experimental study 5-day-old seedlings were treated with 150 mM NaCl, 1mM Spd and 150 mM NaCl supplemented with 1mM Spd for 16 hour. The root and shoot tissues were harvested separately and homogenized in liquid nitrogen for following experimental purposes.

2.3 Chlorophyll , carotenoid and anthocyanin contents

Chlorophyll (chl) and carotenoid (CAR) estimation was performed following the method [12]. Leaf samples (0.3 g) were homogenized with 5 ml of chilled acetone (80% v/v) and centrifuged at 3000 (rpm). The extracts were subjected to record the absorbance at 480, 663 and 645 nm and the pigment concentrations were calculated using the formula given below:

Chl a (mg/g FW) = [{(12.7× (A663) -2.69× (A645)}/1000×W]×V

Chl b (mg/g FW) = [{(22.9× (A645) - 4.69× (A663)}/1000×W]×V

Total Chl (mg/g FW) = [{(20.2×A645) + (8.02×A663)}/1000×W]×V

CAR (mg/g FW) = [A480+ {(0.114×A663)-(0.638×A645)} /1000×W]×V, Where 'W' is the fresh weight of the material and 'V' is the extraction volume.

0.1 g of plant samples were extracted for 24 h with acidified [1% (v/v) HCL] methanol with occasional shaking at 4°C [9]. The anthocyanin content was calculated using extinction coefficient of 31.6 mM−1 cm−1 after recording the absorbance at 525 nm.

2.4 Three dimensional structural analyses and ligand binding site prediction

Three-dimensional homology modelling of each of the rice antioxidative isoenzymes was predicted with the help of I-TASSER server [13]. The predicted structures were further verified based on the percentage of amino acids found outlier region in Ramachandran Plot using RAMPAGE server [14] and necessary refinement of each protein model was executed with the help of MODREFINER server [15]. Final models thus obtained were undergone ligand binding site prediction using COACH server [16]. The 3D structural alignment between proteins pairs of each of the isoenzymes were performed using TM-align: a protein structure alignment algorithm based on the TM-score to explore the structural differentiation [17].

2.5 Enzyme assays

0.2 g of fresh tissue was pulverized in liquid N_2 and extracted with 10 ml of 0.1 M phosphate buffer (pH- 7.0) for CAT assay, with 10 ml of 50 mM potassium phosphate buffer (pH- 7.8) containing 1mM ascorbate for APX assay, with 4 ml of 0.05 M sodium phosphate buffer (pH- 7.0) containing 1% PVP and 4 ml of 0.05 M sodium phosphate buffer (pH- 7.8) for SOD. The homogenates were centrifuged at 10,000 rpm for 30 min at 4°C and the supernatants were used as crude enzyme extracts.

The CAT (EC 1.11.1.6) activity was determined following the method of [18]. Reaction mixtures contained 100 mM potassium phosphate buffer (pH-7), 75 mM H_2O_2, enzyme extract and distilled water. The CAT activity was assayed by measuring the decrease in absorbance at 240 nm for 3 min

due to breakdown of H_2O_2 as a substrate and expressed in mM/g. fr.wt./min using the extinction coefficient of 40 mM−1 cm−1 for H_2O_2 at 240 nm.

The APX (EC 1.11.1.11) activity was measured according to method[19]. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.8), 1 mM H_2O_2, and 0.8 mM ascorbate, enzyme extract and sterile distilled water and made total volume of 2 ml. The reaction started immediately after the addition of crude enzyme extract and decreased in absorbance was recorded at 290 nm for 3 min due to ascorbate oxidation. APX activity was expressed in mM/g. fr.wt. /min using the extinction coefficient of 2.8 mM−1 cm−1 for ascorbate at 290 nm.

SOD (EC 1.15.1.1) activity was quantified following method [20] on the basis of its ability to reduce nitroblue tetrazolium (NBT). Reaction mixture (3 ml) consisted of 50 mM phosphate buffer (pH 7.0), 13 mM methionine, 75 mM NBT, 100 mM EDTA, 0.2 ml of the enzyme extract and 2 mM riboflavin. After the immediate addition of riboflavin the mixtures were shaken and placed 30 cm below two 15-W fluorescent tubes to provide photon flux density of around 40 mmol m-2 s-1. The reaction was incubated for 30 min after which the lights were switched off and the tubes were kept in dark places. Absorbance of the reaction mixture was taken at 560 nm for the reference sets (lacking enzyme) and test sets (with enzyme extract). A blank reaction mixture was made keeping as non-illuminated that served as control and was deducted from absorbance at 560 of above sets. The reference sets showed the maximum absorbance that gradually decreased with increasing volume of the enzyme extract added in test sets. Unit of enzyme activity was taken as the quantity of enzyme which decreased the absorbance reading of test samples producing 50% inhibition in contrasts with the reference sets.

2.6 mRNA isolation and real time PCR

Total RNA from *Oryza sativa* shoots and roots was extracted using POWERPLANT PLANT RNA isolation KIT (MO BIO Laboratories, Inc.). First-strand cDNA was synthesized with SuperScript® III RT Master Mix (Invitrogen, Life Technologies) according to the manufacture's protocol.

The Quantitative real-time PCR (qRT-PCR) was performed on CFX96 Touch™ Real-Time PCR Detection System. The qRT-PCR reaction mixture (20 µl) was prepared with 10 µl 2× iQ™ SYBR® Green Supermix, 1 µl cDNA template, 0.5 µl each of forward and reverse primer (10 µM) and 8 µl of sterile water. The thermal cycling conditions were as follow: pre-denaturation at 95°C for 3 min, followed by 40 cycles at 95°C for 10 s and 60°C for 40 s. After the completion of cycle, the specificity of the amplicon for each primer pair were verified through melting curves analysis at 60–95°C. The rice actin gene was taken as an internal control and the qRT-PCR was carried out with three biological replicates. The specific primers that were used for qRT-PCR represented in **table 2** [21]. The relative gene expression analyses were performed using $2^{-\Delta\Delta t}$ method.

Table 2 Primer list for RT-PCR analysis of rice Antioxidative genes (Kim et al. 2007).

GENE		PRIMER	PCR PRODUCT SIZE(Kb)
Mn-SOD	F	GGAAACAACTGCTAACCAGGA	297
	R	CAATGTACACAAGGTCCAGAA	
Cu/Zn-SOD	F	CAATGCTGAAGGTGTAGCTGA	300
	R	CGAAATCCATGTGATACAAGA	
Fe-SOD	F	TGCACTTGGTGATATTCCACTC	297
	R	CGAATCTCAGCATCAGGTATCA	
Cytosolic APX	F	GACAAGAAACCCTCTGCAGTTT	305
	R	GTAGTCTGCTGGTTCACACTGG	
Thylakoid- APX	F	ATTTTCACTGGACGATGAACCA	320
	R	GGAAGTAGTTGGACTGCAGAGG	
Stromal APX	F	GTCTGGAGCACATACACTTGGA	352
	R	TTAACCGTCCAACGTGAATCCC	
CatA	F	TAAGGCCAGACAATGTCAGATG	206
	R	CAGTGGCATTAATACGCCAGTA	
CatB	F	ACGTCTCAACCTGAAACCAAA	308
	R	GTTCCAGTCTCTAAAGCCATC	
CatC	F	ATCTGGCTCTCCTACTGGTCT	286
	R	AGGAGAAACGTGTCTTCAGGT	

2.7 Endogenous H_2O_2 concentration determination

Endogenous amount of hydrogen peroxide was quantified according to method [21]. 0.5 g of plant samples were ground with 3 ml of 1% (W/V) TCA and centrifuged at 10,000g for 10 min at 4°C. Reaction mixtures were prepared with 0.75 ml of supernatant, 0.75 ml of 10 mM K-phosphate buffer (pH 7.0) and 1.5 ml of freshly prepared 1 M potassium iodide (KI) solution, and the absorbance of the solution was recorded at 390 nm. The concentration of H_2O_2 was calculated from a standard curve prepared with known concentrations of H_2O_2 following above procedure.

2.8 Statistical analysis

Experiments were performed in triplicates and data are represented as means ± standard deviation (SD). The data were subjected to analysis of variance (one-way ANOVA) taking $P < 0.05$ as significant difference. Computation, data analysis and graphics of all the data was employed using Windows Microsoft Excel 2007 software.

3. RESULTS AND DISCUSSION

3.1 Estimation of chlorophyll, carotenoid and anthocyanin content

In IR 36, salt (150 mM NaCl) stress for 16 h reduced significantly ($P<0.05$) by 1.41, 1.29 and 1.28 fold whereas Nonabokra exhibited 1.29, 1.35 and 1.08 fold decrease of the chl a, b and CAR contents respectively than those under the control conditions (**Figure 1**). Exogenous Spd alleviated the salt-induced inhibition of chl a, b and CAR content by 1.13, 1.20 and 1.33 fold in IR 36 respectively, 1.16, 1.06 and 1.08 fold in Nonabokra respectively. The anthocyanin content was significantly ($P<0.05$) variable between the two rice cultivars, IR 36 and Nonabokra. In response to salt stress (150 mM), IR 36 and Nonabokra shoots showed 1.5 and 1.20 times increase in anthocyanin level respectively (**Figure 1)**. Exogenous application of Spd, in conjunction with salt stress further stimulated the anthocyanin amount by 1.22 and 1.20 times than only saline conditions in IR 36 and Nonabokra respectively. Application of only Spd revealed as ineffective in regulation of pigment contents without stress that remain more or less same as control.

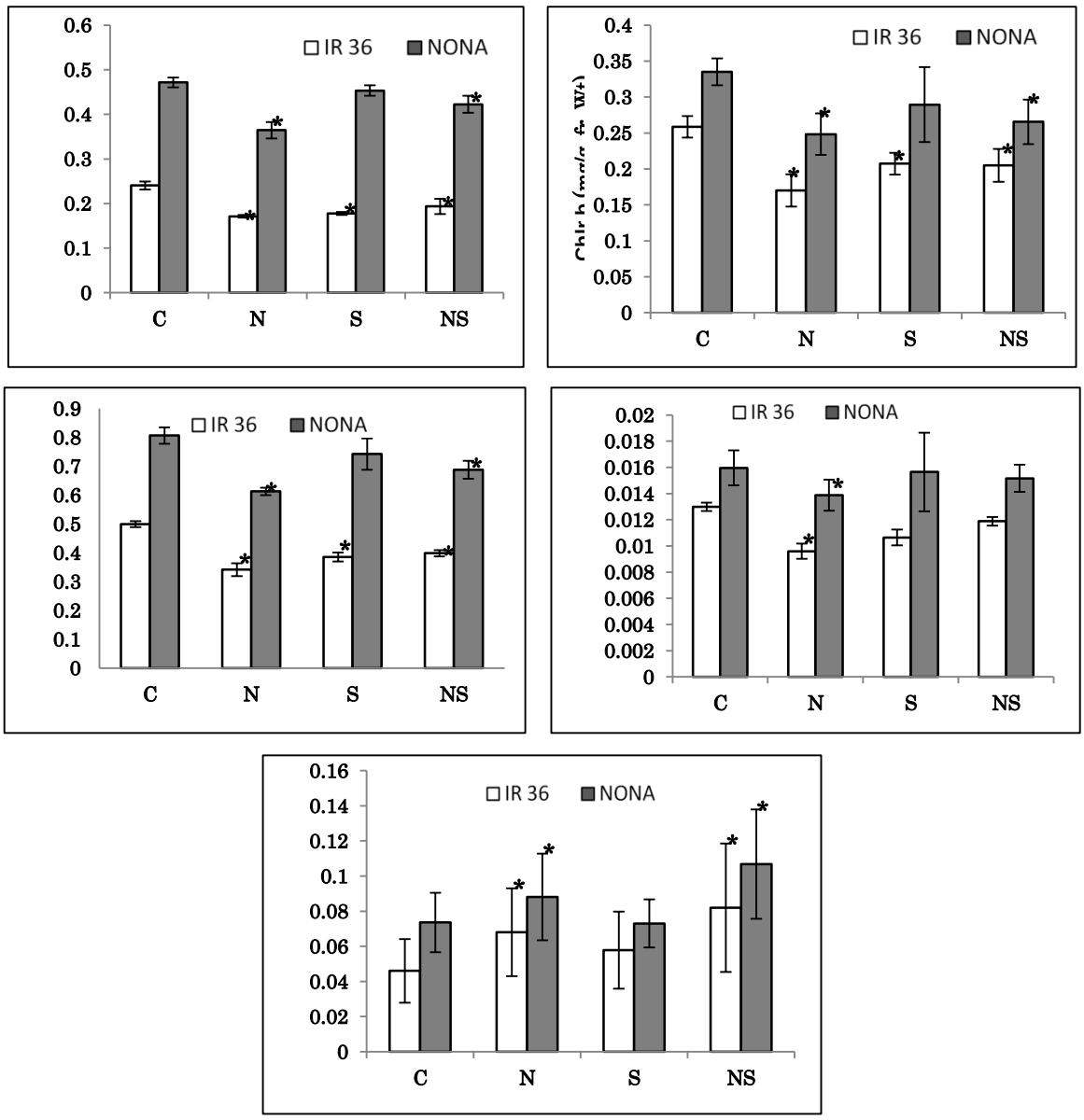


Figure 1 Effects of exogenous spermidine (1 mM) on chlorophyll, carotenoid and anthocyanin content in the roots and shoot tissue of 5-day rice seedlings of two rice cultivars IR-36 (Salt sensitive) and Nonabokra (salt tolerant) under 16 hour treatment of 150 mM NaCl. Data are the mean value (n = 3) ± SE. The SE in each case is represented by the vertical bar in each graph. Asterisks indicate significant differences from the corresponding control (*P<0.05). C=Control, N= 150 mM NaCl, S= 1 mM Spd, NS= 150 mM NaCl and 1 mM Spd.

3.2 3D structure and ligand binding site of each isoenzymes

TIGR Rice Genome Annotation Project revealed that there are three isoforms each of CAT [*catA*, *catB* and *catC*], SOD [*Cu/Zn sod*, *Fe-sod* and *Mn-sod*] and APX [*cytosolic apx (cyt apx)*, *thylakoid apx* (*thyl apx*) and *stromal apx (strml apx)*] enzymes. We have modelled 3D structure of each of the isoenzymes using I-TASSER server and represented in **Figure 2.** An optimal superposition of each of the isoenzymes pairs produced TM-score higher than 0.5 that are assumed to be validated structural similarity (**Table 3**). To further explore the functional specifications, we have performed the COACH analyses to execute the 3D view of ligand protein interaction of each of the isoenzymes (**Figure 2**).

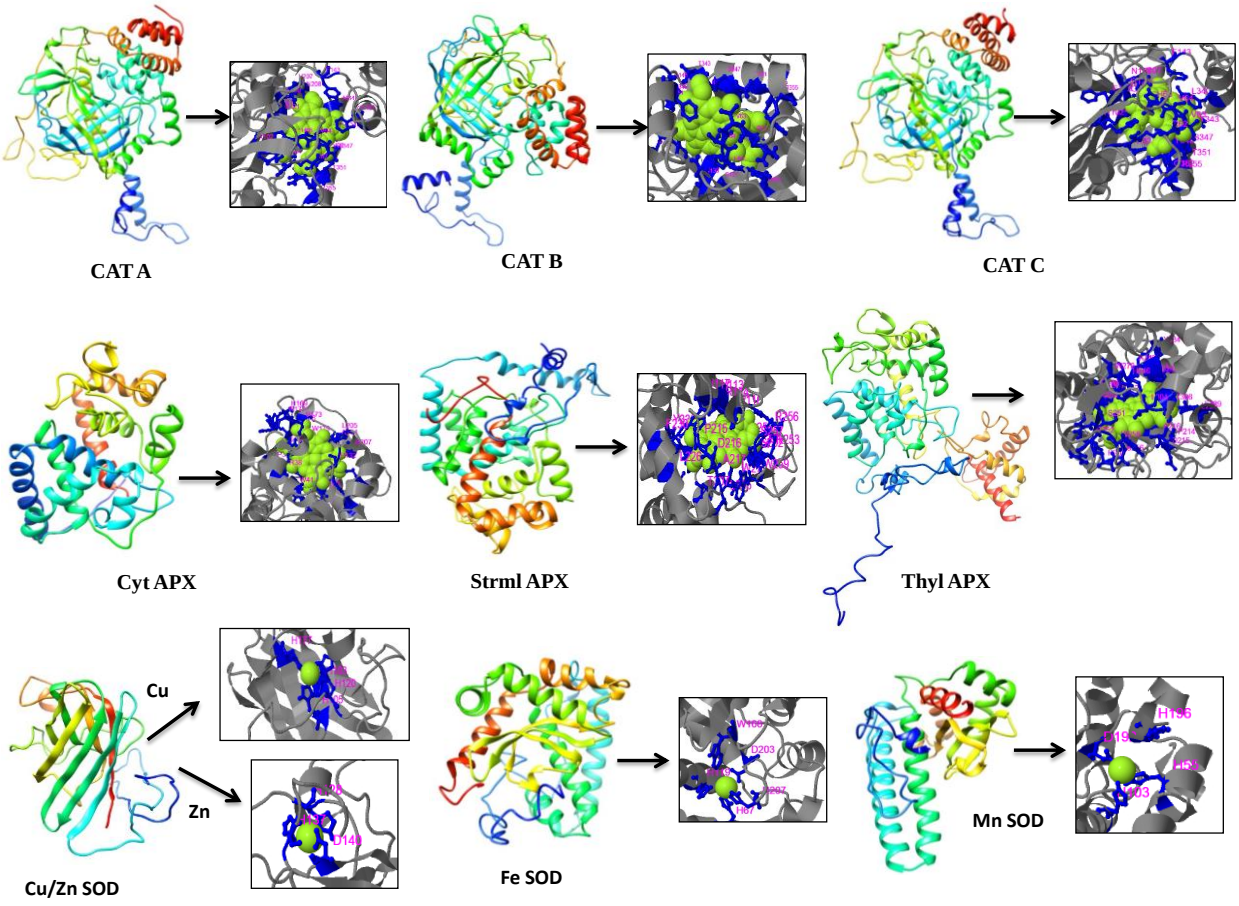


Figure 2 The 3D view and ligand-protein interaction site of each of the rice antioxidant isoenzymes derived from COACH server.

Table 3 3D alignments score of each isoenzymes pair of proteins using TM-Align.

Gene Name	Protein 1	Protein 2	TM-Score
	CAT A	CAT B	0.91025
CAT	CAT A	CAT C	0.96965
	CAT B	CAT C	0.90877
	Cyt APX	Thyl APX	0.933320
APX	Cyt APX	Strml APX	0.93830
	Thyl APX	Strml APX	0.61218
	Mn-SOD	Cu/Zn-SOD	0.31324
SOD	Mn-SOD	Fe-SOD	0.82321
	Cu/Zn-SOD	Fe-SOD	0.28582

An algorithm for protein structure alignment and comparison

*** Based on statistics**

*** 0.0 < TM-score < 0.30, random structural similarity**

***0.5 < TM-score < 1.00, in about the same fold**

The detailed statistical parameters, most probable ligand name and the ligand-binding residues in the protein 3D model were illustrated in **Table 4**. There are no major differences in tertiary structure and ligand binding sites among CAT A, B and C isoenzymes as derived from the high Tm-Score > 0.9. But the APX isoenzymes passed through utmost divergence in their ligand binding sites though cyt APX shared maximum structural similarity with the other two isoenzymes. Maximum structural divergence was found in Cu/Zn SOD protein that has shown Tm-Score of 0.31324 and 0.28582 with its isoenzymes Mn-SOD and Fe-SOD respectively.

Table 4 Ligand binding site prediction of each of the antioxidant isoenzymes using COACH.

Gene Name	C-score	TM-score	IDEN	Cov	BS-score	Ligand Name	Predicted binding site residues
CAT A	0.79	0.931	0.481	0.963	1.77	HEME	62,63,64,65,102,121,136,137,138,143,148,151, 207,208,324,340,343,344,347,348,351,352,355
CAT B	0.75	0.878	0.461	0.931	1.77	HEME	62,63,64,65,102,121,136,137,138,143,148,151, 207,208,324,340,343,344,347,348,351,352,355
CAT C	0.79	0.941	0.478	0.970	1.79	HEME	62,63,64,65,102,121,122,123,136,137,138,143,151, 207,208,324,340,343,344,347,348,351,352,355
Cyt APX	0.91	0.988	0.824	1.000	1.91	HEME	34,38,41,132,134,141,145,159,160,162,163,165,166, 167,168,169,172,173,179,205,207,235
Thyl APX	0.87	0.571	0.865	0.575	1.91	HEME	109,112,113,116,214,215,216,225,229,242,243,245, 246,249,250,251,252,255,256,258,278,304,306,334
Strml APX	0.86	0.761	0.840	0.766	1.91	HEME	110,113,114,117,215,216,217,226,230,243,244,246, 247,250,251,252,253,256,257,259,279,305,307,335
Mn-SOD	0.91	0.859	0.783	0.875	1.81	MN	55,103,192,196
Cu/Zn-SOD	0.88	0.716	0.882	0.725	1.55	ZN	128,137,140
	0.85	0.719	0.870	0.730	1.98	CU	103,105,120,177
Fe-SOD	0.76	0.738	0.448	0.753	1.48	FE^{2+}	67,119,168,203,207

C-score: It is the confidence score of predicted binding site. C-score values range in between [0-1]; where a higher score indicates a more reliable prediction.

IDEN: is the percentage sequence identity in the structurally aligned region.

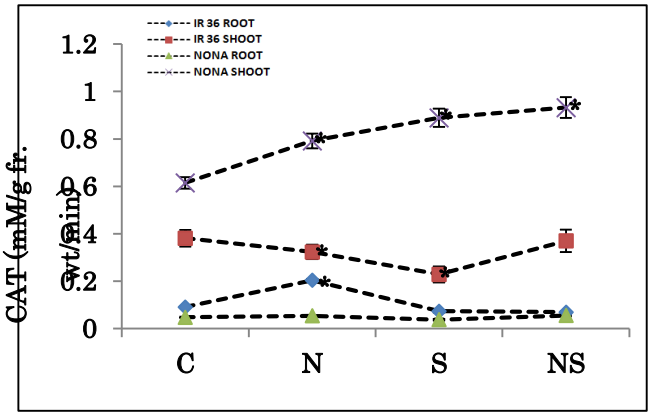
Cov: It represents the coverage of global structural alignment and is equal to the number of structurally aligned residues divided by length of the query protein.

BS-score: It is a measure of local similarity (sequence & structure) between template binding site and predicted binding site in the query structure. Based on large scale benchmarking analysis, we have observed that a BS-score >1 reflects a significant local match between the predicted and template binding site.

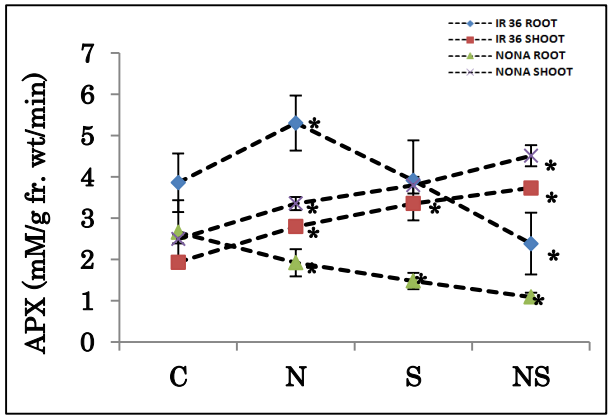
3.3 Antioxidative enzymes activities

We have presented here the tissue specific activities of antioxidative enzymes in SSC (IR 36) and STC (Nona) in response to short term exposure of salt stress (16 h) in presence and absence of external Spd (**Figure 3)**. Statistically significant increased in activities of CAT, APX and SOD were observed in IR 36 root and Nonabokra shoot. In IR 36 root, CAT, APX and SOD activity increased by 2.62,

1.37 and 2.47 fold over control respectively, while the corresponding increase in Nonabokra shoot were 1.3, 1.34 and 1.33 fold over control (**Figure 3A-3C)**. The salinity induced activities of CAT, APX and SOD were further stimulated by 1.17, 1.34 and 1.4 times respectively at the significant level ($P<0.05$) with the application of exogenous Spd in Nonabokra shoot. However, the trend was opposite for the IR 36 root where Spd treatment reduced the salt induced activities of antioxidative enzymes significantly ($P<0.05$) and tried to bring it to the control level. APX and SOD activities were increased significantly in IR 36 shoot by 1.45 and 1.18 times respectively with compared to control while the corresponding increase by 1.34 and 1.15 fold in activities of both the enzymes remain consistent in presence of Spd treatment with saline condition over only salt induction. Nonabokra root showed no significant changes ($P<0.05$) in response to short term exposure to salt treatment in presence or absence of externally applied Spd with respect to CAT activity. In case of APX and SOD activities, significant decrease was observed by 1.38 and 1.92 fold respectively over control in Nonabokra root, and corresponding salt-stimulated reduction was further induced by 1.75 and 1.53 fold in cumulative Spd and salt treated plant tissue over the salt treatment.



[A]



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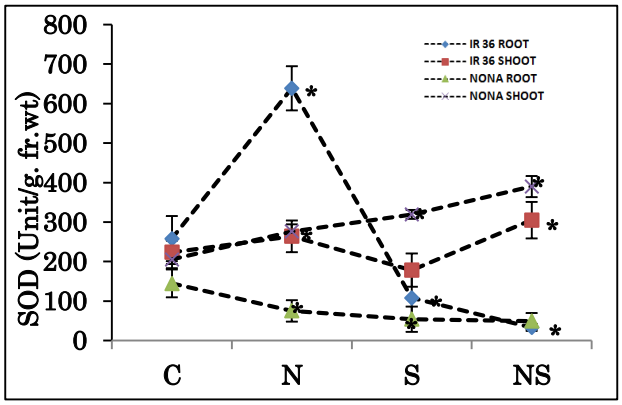
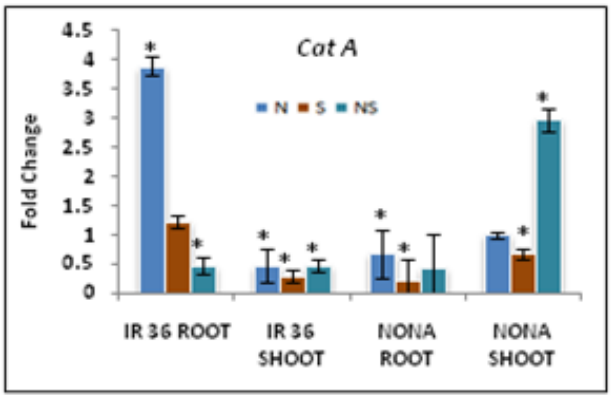


[C]

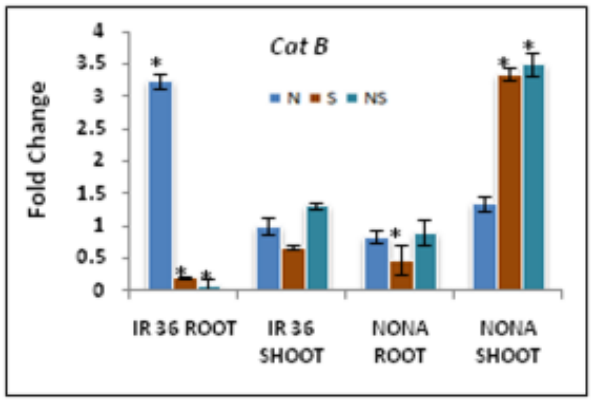
Figure 3 Effects of exogenous spermidine (1 mM) on the activities of CAT [A], APX [B] and SOD [C] in the roots and shoot tissue of 5-day old rice seedlings of two rice cultivars IR-36 (Salt sensitive) and Nonabokra (salt tolerant) under 16 hour treatment of 150 mM NaCl. Data are the mean value (n = 3) ± SE. The SE in each case is represented by the vertical bar in each graph. Asterisks indicate significant differences from the corresponding control (*P<0.05). C=Control, N= 150 mM NaCl, S= 1 mM Spd, NS= 150 mM NaCl and 1 mM Spd.

3.4 Expression profile of each of the antioxygenic isoenzymes

To validate our physiological and biochemical analysis, we have performed real time PCR amplification to evaluate the tissue specific mRNA accumulation of each isoforms of antioxidative enzymes in both the cultivars**.** In this study we have presented expression profile of each isoforms to enlighten the cumulative contribution on the enzyme activities at the biochemical level **(Figure 4A-4J)**. IR 36 root displayed maximum level of transcript accumulation of *catA*, *catB* and *catC* genes during salt stress, but exogenous Spd application down-regulated those genes in presence or absence of salt stress. This trend is consistent with the *cyt apx*, *Cu/Zn sod*, *Fe-sod* and *Mn-sod* isoforms. Up and down regulation in transcript levels of each of the antioxidative enzymes were reflected in increased and decreased enzyme activities in IR 36 root. SOD and APX isoforms were unregulated in shoot tissue of both the cultivars in saline conditions than control. Exogenous Spd further enhanced their expression at the time of recovery from salt stress. CAT isoforms were decreased in response to salt stress in IR 36 root but recover to an increased level with the help of Spd that exhibited congruency to its CAT enzyme activity. Nonabokra shoot showed maximum mRNA abundance of CAT isozyme genes in response to Spd treatment with or without salinity. No significant fold change was observed at the transcript level of each of the isozymes in Nonabokra root irrespective of the salt stress in presence or absence of exogenous Spd.



[A]



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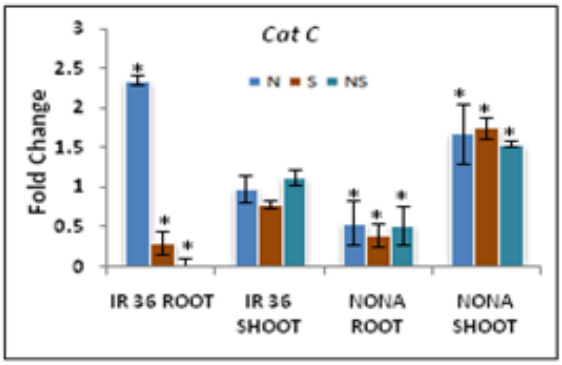
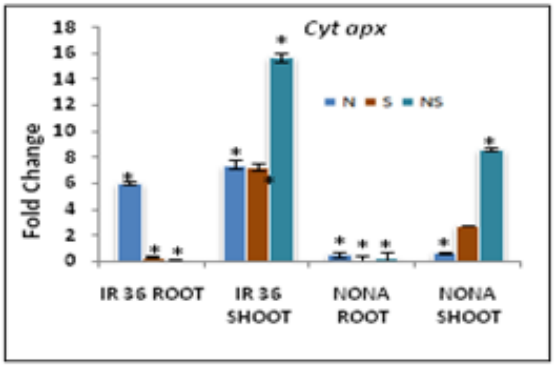


[C]



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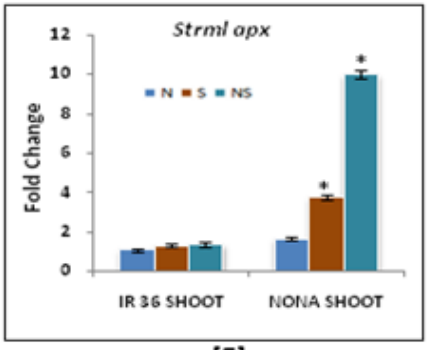

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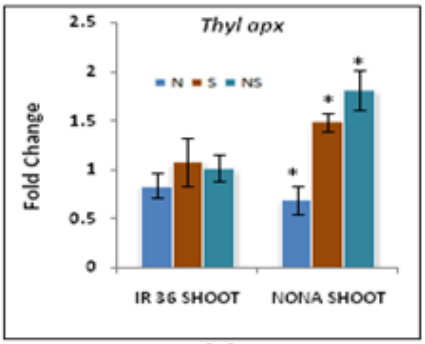
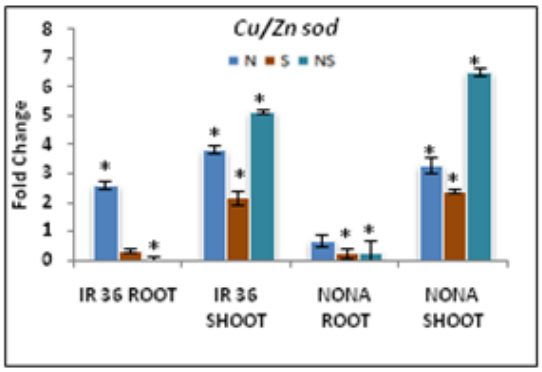
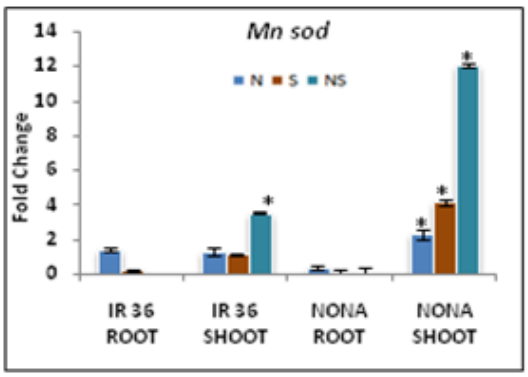


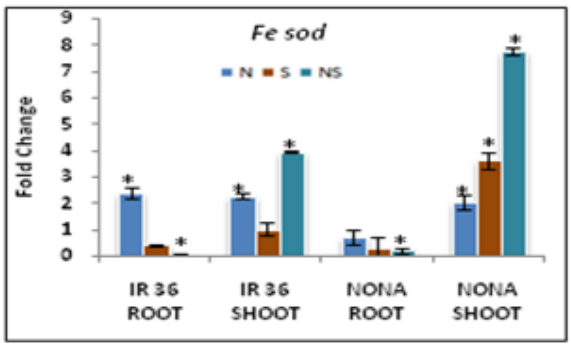
[F]



[G]



[H]



[I]

Figure 4 Effects of exogenous spermidine (1 mM) on the fold change of each isoenzymes of CAT [A-C], APX [D-F] and SOD [G-I] under 16 hour treatment of 150 mM NaCl in comparison to control in the root and shoot tissues of 5-day old rice seedlings of two rice cultivars IR-36 (Salt sensitive) and Nonabokra (salt tolerant). Data are the mean value (n = 3) ± SE. The SE in each case is represented by the vertical bar in each graph. Asterisks indicate significant differences from the corresponding control (*P<0.05). N= 150 mM NaCl, S= 1 mM Spd, NS= 150 mM NaCl and 1 mM Spd.

3.5 H_2O_2 quantification

The hydrogen peroxide (H_2O_2) content increased significantly in rice seedlings under saline conditions **(Figure 5)**. Roots and shoots of IR 36 accumulated H_2O_2 content by 5.26 and 3.37 fold whereas Nonabokra roots and shoots have shown 1.87 and 2.45 fold rise with compare to control. Treatments with 1 mM Spd in presence of salt significantly suppressed salinity-induced H_2O_2 by 8.56 and 1.81 fold in root and shoot of IR 36 respectively whereas Nonabokra root and shoot showed 1.21 and 1.55 fold reduction after 16 h compared to salt stress. The exogenous application of Spd without saline condition was unable to alter the H_2O_2 content significantly, as compared to the control. These observations demonstrated that exogenous Spd restrict the ROS level thereby protecting rice seedlings from the damage of salt stress.

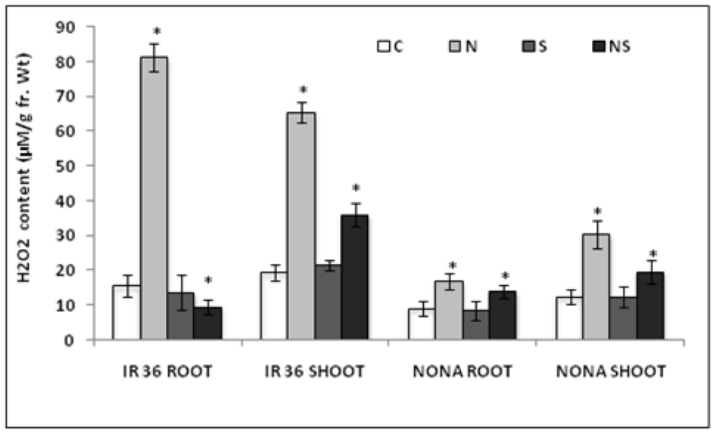


Figure 5 Effect of exogenous spermidine (1 mM) on H_2O_2 generation in the roots and shoot tissue of 5-day rice seedlings of two rice cultivars IR-36 (Salt sensitive) and Nonabokra (salt

tolerant) under 16 hour treatment of 150 mM NaCl. Data are the mean value (n = 3) ± SE. The SE in each case is represented by the vertical bar in each graph. Asterisks indicate significant differences from the corresponding control (*P<0.05). C=Control, N= 150 mM NaCl, S= 1 mM Spd, NS= 150 mM NaCl and 1 mM Spd.

Alteration of endogenous PA concentrations was greatly acknowledged in alleviating salt stress response which in turn is dependent on plant species and cultivars, duration and intensity of stress treatment, plant organ and developmental stage of tissues [23]. In our work, we have investigated the outcome of the short term exposure of salt stress and its ameliorative response in presences of exogenous Spd. In our findings, we exhibited that salt stress instigated substantial reduction in the pigment contents (Chl, CAR and anthocyanin) in rice cultivars, but SSC respond more than STC towards salinity. The decline in Chl content inevitably took place during salt stress due to toxic ion accumulation and singlet oxygen production that has been reflected in the antenna heterogeneity in photosystem II (PSII) and ultrastructure of chloroplasts leading to the decrease in rate of photosynthetic electron transport. The CAR and anthocyanin is a predominant phenolic compound specifically required for healing oxidative stress by non enzymatic pathways. Salt stress induced reduction in CAR and anthocyanin content facilitate the accumulation of reactive oxygen species in cellular environment. Application of exogenous Spd to the salinized nutrient solution endow with tolerance in the seedlings of rice, tomato, ginseng and cucumber plants by preventing chlorophyll degradation, enhancing net photosynthetic rates, actual photochemical efficiency of PSII, plant growth (fresh weight, dry weight, shoot height) compared to the control [24,25,26]. Our finding also revealed, the supplementation of Spd rescued the physiological parameters of salt injured rice plants in terms of growth and pigment contents that have found congruency with the previous reports and established a strong correlation between Spd application and prevention of growth retardation and loss of pigment contents suggesting their possible role as protective shield of photosynthetic proteins and pigment protein complexes against protease action under stress conditions.The ROS signaling network is intricately coordinated and highly conserved among aerobic organisms regulating a wide range of biological processes such as growth, development, and responses to biotic and/or abiotic stimuli [27]. During ROS signaling, non-toxic levels is maintained in a delicate balancing act between ROS production, including ROS-producing enzymes and the metabolic counter-process involving ROS-scavenging pathways [28]. Plant acquired several defensive mechanisms to combat with oxidative stress generated during saline environment. CAT-SOD-ASC cycle, a most important part of the ROS-scavenging network in plants is associated with PAs anabolism and catabolism [2]. The whole cycle attributes to controlling ROS levels, as well as the redox state of ascorbate, glutathione and pyridine nucleotides, metabolites involved in cellular redox homeostasis [29]. In this study we have focused on three antioxidative enzymes, CAT, APX and SOD, the main enzymes that construct the essential node of that cycle. Our data demonstrated, salt stress elevated the activities of SOD and

APX in root and shoot tissue of IR 36 (SSC) and shoot tissue of Nonabokra (STC), although the overall increase was much higher in roots of IR 36, as root is the primary perceptive organ of environmental cues. CAT enzyme showed increased activity in IR 36 root and Nonabokra shoot but it was reduced in IR 36 shoot. Significant increased amount of H2O2 (product of SOD activity) content generated in both the rice cultivars was imitated with increasing SOD activity at short-term NaCl stress when compared to the control plants, which subsequently necessitates the stimulation of H_2O_2 eliminating enzymes (CAT and APX) activities. The improved salt tolerance were highly correlated with increased activation of antioxidant enzymes in Arabidopsis, rice [30], wheat, tomato [24], soybean, maize [31], which is in accordance with our observations. Even many previous reports on genotype dependent accumulation of H_2O_2 content and differential response of antioxidative enzymes in salinity have been done in rice cultivars [32,33]. We have extended their work in a tissue specific manner to further elucidate the contribution of root or shoot towards enhancement of plant defense system in salinity. Surprisingly Nonabokra root exhibited no significant differences in activities of antioxidative enzymes during salt stress. This observation made us put our view regarding the tolerant cultivar that short duration of salt exposure could not accumulate the toxic Na^+ ions as they may be sequestered in the vacuole of root. On the contrary, increased activities of antioxidative enzyme in Nonabokra shoot suggesting the fact that short term salt stress was enough to enable the root to send a stress signal to shoot so that it can induce its stress machinery to combat against upcoming salt induced damage.

In our previous review, we have enlightened the fact that plant polyamines counteract the salinity induced ROS by acting as free radical scavengers, or activating antioxidant enzymes, thus maintaining ROS homeostasis [2]. We have performed rigorous scanning on the effect of exogenous Spd in regulating the antioxidative enzyme systems to make a correlation with PA. We observed that exogenous Spd further increased the salt induced activity of CAT, APX and SOD in shoot tissue of both the cultivars. These results corroborate previous reports of high CAT, APX and SOD activity in rice [9] and ginseng seedlings [25]. In addition to that, exogenous Spd was shown to have mitigating effect on salt induced cellular and macromolecular damage by maintaining the H_2O_2 production. It has been found that exogenous Spd recuperated the inhibitory effect on growth of tomato plant and restrained the accumulation of H_2O_2 and O^{2-}. resulted from saline-alkaline stress, again supporting our observation [34]. Our data demonstrated that IR 36 root showed maximum reduction of salt-stimulated antioxidative enzymes activities in presence of exogenous Spd which is positively linked with low H_2O_2 production. The probable recovery mechanism lying behind this event might be that being a polycationic charge of Spd enable them to bind to negatively charged plasma-membrane and block the root ion channels thus preventing the inward Na^+ and K^+ currents (especially Na^+ currents) in root epidermal and cortical cells [35]. However, the changes in the intracellular environment of root in salt+Spd treatment provide a stress cues to the shoot to turn on its stress relieving machinery

that was substantiated with increased activities of CAT, APX and SOD activities.

At the molecular level, CAT, APX and SOD enzymes have multiple molecular forms (isoenzymes) in rice that may passed through different regulatory mechanism to act upon stress-induced ROS to minimize stress injury and can be used as biomarkers of various environmental stresses due to their differential response to various environmental stress [21]. Among the three isoforms of SOD, *Cu/Zn-sod* is localized in all subcellular fractions, *Mn-sod* was found in the mitochondrial and Fe-sod in the chloroplast [36]. The isoforms of APX existed in thylakoid, stroma and cytosol whereas plants CATs have been grouped into three different classes, *catA, catB* and *catC* [21]. In our study we have made an attempt to scrutinize the tissue specific transcript abundance during short-term salt stress and the recovery effect of exogenous Spd regulating the expression profile of genes encoding each of the above antioxidant isoenzymes. SOD and APX isoforms have shown incongruity in their trend of expression patterns depending on the plant species, tissues, stress treatment, stress duration and differences in the localization of ROS in subcellular compartments [37,38]. In, our observations, *Cu/zn-sod* and *Fe-sod* were found to more effective in short-term salinity than *Mn-sod* in IR 36 root and shoot and Nonabokra shoot. On the other hand, *cyt apx* was more pronounced in IR 36 cultivar but *strml apx* and *thyl apx* remained unchanged in both the cultivars. External Spd treatment was found to be more effective in elevating the abundance of APX and SOD isoforms in shoot tissue of IR 36 and Nonabokra, but root tissues remained ineffective for the same. In case of CAT isoforms, IR 36 root responded more efficiently to salt resembling with the previous report of up-regulation of *catB* and *catC* in 24-72 h salt stress [39], while the other tissues showed no marked change in their expression pattern. Applications of Spd markedly up-regulated the *catA* and *catB* expression in Nonabokra shoot that contribute to the elevated enzyme activity in this tissue. Our finding found congruency with the previous observations on the inducing effect of PAs on *Cu/Zn-sod, Pgcat, Pgapx* [25,40].

In the present study we have performed high throughput screening to establish the gene–protein–enzyme activity responses in contributing defence to salinity stress in presence or absence of exogenous Spd in different tissues of rice genotypes. The difference in secondary and tertiary structures and the divergence of amino acid sequences among the isoenzymes of rice antioxidant gene family suggest that each of the protein might have gained functional specification at the subcellular level depending of the stress type, intensity, duration that are need to be explored by the future scientists. The cumulative recovery capability of Spd were dependent on tissue types and genotypes of rice seedling and highly correlated with difference in salinity tolerance in terms of pigment content, antioxidative ability, H_2O_2 content. Our present investigations provide first time information regarding the role of exogenous Spd on regulating the expression profiles of each of antioxidative isoenzymes in rice seedlings that are tissue and genotype specific. Moreover, the accumulative degree of expression of each of the isoenzyme genes are positively reflected in the total enzymatic activities

at the biochemical level and differentially attributed to salt tolerance in the plants. These data can be used as first hand information to pursue in the future experiments on how the PAs would be beneficial for relating gene–protein–enzyme activity responses to different environmental stress.

CONFLICT OF INTEREST

The authors have no conflict of interest.

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