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An investigation on the role of salicylic acid alleviate the saline stress in rice crop (Oryza sativa (L))

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ABSTRACT

Among the different naturally occurring plant hormones Salicylic Acid (SA) is one of the important signal molecules. The current study was carried out to investigate whether foliar spray of SA could improve the different parameters and help to alleviate the saline stress in rice seedlings. Saline stress (100 mM NaCl) significantly reduced the photosynthetic and total protein content. Increased NaCl concentration exhibited a significant increase in antioxidant enzymes activity. Plants grown under high saline stress showed more accumulation of ROS with DAB staining and exhibited the fragmented nuclei by DAPI staining. Foliar spray of SA showed the improved photosynthetic and protein content, reduced antioxidant enzymes activity, lower accumulation of ROS and intact nuclei. Further, expression of Catalase-1 (CAT-1), Superoxide dismutase (SOD) and Glutathione Peroxidase (GPX) genes showed upregulation during NaCl treatment and coincide with antioxidant enzymes activities. Expression of Mitogen activated protein kinases-1 (MAPK-1), transcription factor WRKY53, Bax Inhibiotr-1 (BI-1), and nine Autophagy Related Genes (ATGs) were showed upregulation during NaCl treatment. Foliar spray of SA induced the expression of different genes in control and NaCl treated rice seedlings and suggested that SA helpful to alleviate the saline stress. The study suggested that SA is a potent signaling molecule not only with promotive effect on plants but may also be helpful to reduce the toxic effect of saline stress. Furthermore, study concludes that saline stress is regulated by multiple pathways including SA, MAPK1, Programmed Cell Death (PCD) and autophagy. These pathways may help to cope up with saline stress in rice seedlings.

1. Introduction

In recent decade worldwide agriculture experiencing the reduced growth and productivity due to the greater impact of abiotic stress factors including heat, cold, drought, salinity, nutrient deficiency and metal ion stress. It has been observed that these stresses reduced yield more than fifty percent of the total yield of major crops. Among different abiotic stress salinity is one of the most challenging and potential threat to the current agriculture. It reduced the plant growth and yield by interfering the physiological, biochemical, cellular and molecular mechanism in plants (Akbarimoghaddam et al., 2011). Saline stress affects the plant growth from the seed germination to vegetative and reproductive development. It causes ion toxicity, osmotic stress, oxidative stress, nutrient deficiency and poor water and nutrient uptake from the soil in plants.

Rice is a staple food crop of the world and more than 50% population in Asia depends on rice for their food consumption. India is the second largest rice producer worldwide (USDA). Rice is a more prone crop to saline stress at vegetative stage than the reproductive and grain filling stage (Razzaque et al., 2017). Hence, it is important to develop the salt tolerant rice cultivars with improved traits. Salt tolerance is a complex mechanism due to varying responses at cellular and molecular level. Therefore, it is an urgent need to develop different methods and strategies to eliminate the toxic effect of saline stress. Salicylic acid (SA) is a wellknown signaling molecule and critical for plant immunity (Durrant and Dong, 2004). It is a phenolic compound and secondary metabolites of different prokaryotes and eukaryotes including plants. It has been well established that SA has a regulatory role in plant growth and development as well as it provides the defense from various abiotic and biotic stresses including salt stress in plants (Idrees et al., 2011; Jini and Joseph, 2017). SA Modulate the defense responses by improving photosynthetic activity, antioxidative metabolism, vincristine and vinblastine accumulation in *catharanthus roseus* under water stress (Idrees et al., 2011). SA also improves the drought and salinity tolerance in barley (Fayez and Bazaid, 2014). The signaling network of SA involved with different well established pathways.

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The autophagy or self-eating is a degradation pathway of cellular components and plays significant role in stress tolerance. Different autophagy related genes encoding autophagy proteins have been discovered and expressed at different steps of autophagy mechanism. Earlier, it has been reported that autophagy induces during nutrient starvation, biotic and abiotic stresses in plants (Wu et al., 2017). Previously, we have reported the role of autophagy during salt stress and concluded that autophagy process involved in salt tolerance mechanism with autophagic cell death process (Khan and Hemalatha, 2016). The present study aimed to analyze the role of SA at the biochemical, cellular and molecular parameters as well as in alleviation of saline stress. Furthermore, to identify that whether autophagy and PCD pathways are also interconnected with SA signaling mechanism and co-regulated during saline stress in rice seedlings.

2. Materials and methods

2.1. Plant material

The seeds of rice (*Oryza sativa* L.) cultivar CO-51 were procured from Krishi Vigyan Kendra, Tamilnadu Veterinary and Animal Sciences University (TANUVAS), Chennai, India. Firstly, seeds were washed with distilled water to remove dust contaminants and sterilized with 1% sodium hypochlorite solution. Sterilized seeds were soaked for 24h in distilled water and placed on petriplates for germination.

2.2. Treatments

The seedlings were grown in petriplates supplied with distilled water. After seedlings attain the height of 2 cm were given NaCl treatment to induce saline stress by the gradual method to reduce the osmotic shock (25 mM after 24 h until the final concentration of NaCl reached 100 mM). A uniform concentration 0.5 mM of SA was applied as foliar spray to rice seedlings. The treatments were as follows Control: water, NaCl: 100 mM, SA: 0.5 mM and NaCl + SA: 100 mM + 0.5 mM.

2.3. Biochemical analysis

The Total chlorophyll and carotenoids content in fresh leaves were estimated by (Mackinney, 1941; Maclachlan and Zalik, 1963). Anthocyanin content (Laxmi et al., 2006). Protein contents (Lowry et al., 1951). Catalase (CAT) activity (Idrees et al., 2011; Chandlee and Scandalios, 1984). Superoxide Dismutase (Beauchamp and Fridovich, 1971). Peroxidase activity (Idrees et al., 2011).

2.4. Cell biological analysis

Reactive oxygen species (ROS) production was determined by staining of leaves with DAB (3, 3′-Diaminobenzidine) and with DAPI (4′, 6-diamidino-2-phenylindole) to study the nuclear fragmentation by the method of (Gadjev et al., 2004).

2.5. Molecular analysis

2.5.1. RNA isolation and real time PCR analysis for gene expression

To isolate the total RNA from rice leaves Trizol reagent (Invitrogen) was used according to manufacturer's protocols. Purity of RNA was estimated at absorbance 260–280 nm. The amino acid sequences encoding CAT1 Forward Primer (FP) ACTTCGACAGGGAGCGTAT, reverse primer (RP) AGGTGAGGTGGGTGATGT, GPX (FP) GCATCCCAATGTG GCTTAAC, (RP) GCCTTGGACCTTGTACTTCT, SOD (FP) TGGCGCTCC GCACGCTG, (RP) TGGCGACGTAGGTGGCGTGG, BI-1 (FP) TCCCACCT CAAGCTCGTTTA, (RP) ATAGTCAACATCCCGCCGAT, WRKY53 (FP) GGGTGCCCAAGTTCAAGT (RP) TACCTCTGAGCCGGGATT and ATG1 (FP) ATTGCGGACTTTGGATTTGC, (RP) AGCTTGCATGACTTCTGGAG, ATG3 (FP) GGTGAAGGCTGGCTTGCCACACA, (RP) TCTGCTTGTGCTG CCCAGATC, ATG4 (FP) TGGCAAACCCTTGTCCGCAC, (RP) TAGCTCA GTGGCGCGAGAACAG, ATG6 (FP) GGGTTCTCTACTGGTTTATCGG, (RP) GGAAGACTGTGCTACGTGTAAAG, ATG7 (FP) GATCGTGACAGC CCAAAAGCA, (RP) GACCAAGTGGACCCTCACTGC, ATG8 (FP) GTTGG AGATGGCCAGGACTTC, (RP) CAATCCAGCACAGGGACATGC, ATG9 (FP) ATTGAAGAAGCAAGGGGCTGGGACTCT, (RP) CTGCGGGAGATC AGCAAAAGGAAGA, ATG10 (FP) CTGGCGATGGAAGCCTTGCTGC, (RP) TCCTACTGCCTGGCCAACCACAG, and ATG13 (FP) CGCCAACCA ATCCTACAACTAC, (RP) CAACATCATCCACAGCAAAGGG, Actin-1 (FP) GGACTCTGGTGATGGTGTCAGCCA, (RP) GAGCTGGTCTTGGCAGTCT CCA of rice were retrieved from Genbank. The primers were designed and purchased from Integrated DNA Technologies (IDT) USA.

The CFX 96 Touch Real-Time Detection System (Bio- Rad, CA, and USA) was utilized to examine the expression of genes and SYBR Green Supermix (Biorad) for monitoring double-stranded DNA synthesis. The normalized gene expression was calculated with ΔΔ CT method of (Livak and Schmittgen, 2001). To normalize the expression in control and treated samples *O. sativa* Actin-1 was used as a reference gene.

3. Results and discussion

3.1. Biochemical constituents

The biochemical parameters such as chlorophyll *a*, *b*, total chlorophyll, total carotenoids, anthocyanin (Fig. 1) and total protein (Fig. 2) are affected significantly under saline stress. In all four treatments, SA alone significantly increased the Chl. a, b, total chlorophyll and carotenoids contents in rice seedlings. Plants showed the significant decrease in pigments after NaCl treatment while it was increased when SA was applied with NaCl. The total protein content was estimated and represented in (Fig. 2). Protein content significantly increased in SA treatment followed by NaCl + SA and lowest in NaCl treatment. The antioxidant enzymes including CAT, SOD and POX showed maximum activity in NaCl treated plants (Fig. 3a–c). Plants treated with SA showed minimum antioxidant activity as compared to control.

Since, long time the specific role of signaling molecules in enhancing crop productivity has been practiced. However, salicylic acid as a plant growth regulator and alleviation of stress conditions for sustainable crop productivity has received much attention in recent years. Plants have evolved a complex mechanism to counter the environmental stress such as salinity which causes the NaCl toxicity and lowered water potential (Munns and Tester, 2008). Salt stress induced the

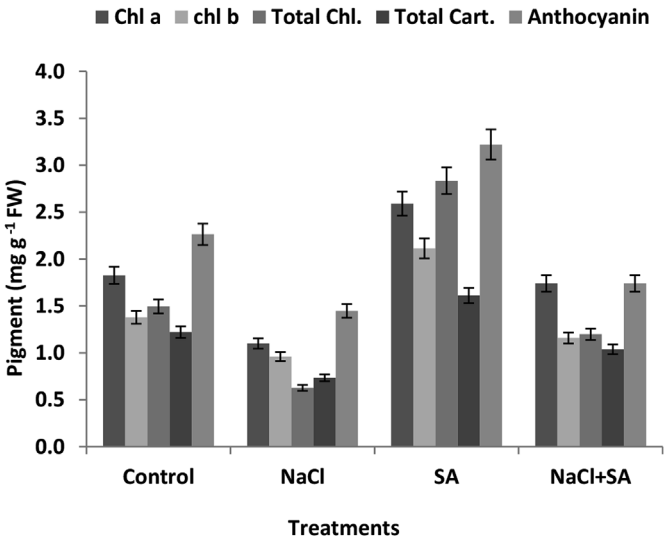

Fig. 1. Effect of NaCl, SA, NaCl + SA on Chlorophyll *a*, *b*, total Chlorophyll, carotenoids and total anthocyanin content in *O. sativa* seedlings (CO-51). The standard errors are mean of three independent experiments.

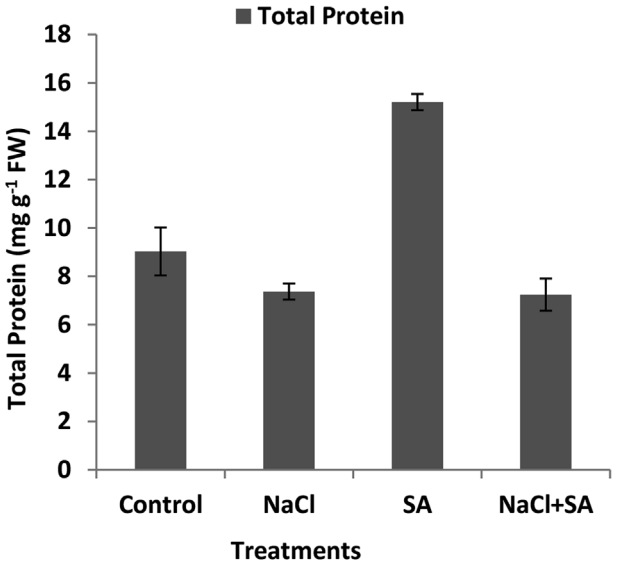

Fig. 2. Effect of NaCl, SA, NaCl + SA on Total protein content in *O. sativa* seedlings. The standard errors are means of three independent experiments.

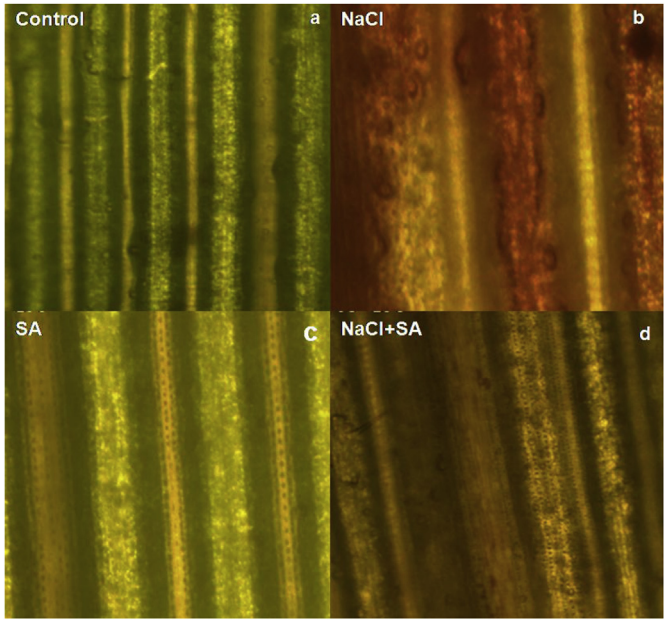

Fig. 4. Leaves of *O. sativa* treated with NaCl, SA, NaCl + SA and stained with DAB to detect ROS production under light microscope with 40X magnification.

inhibition of plant growth specifically by ion toxicity, impaired Na^+, Cl^- ion homeostasis, ROS accumulation and closure of stomata (Gunes et al., 2007; Daneshmand et al., 2010). Salt stress reduced the photosynthetic machinery due to change in photosynthesizing tissues, toxicity of Na^+ and Cl^- ions and increased ROS production that leads to inhibition of photosynthetic pigments (Nazar et al., 2015; Ram et al., 2017). In the present study salt stress (100 mM NaCl) reduced the photosynthetic pigments (Chl. a, b total chlorophyll and carotenoids, anthocyanin) and protein content (Figs. 1 and 2). It was observed that foliar application of salicylic acid (0.5 mM) significantly enhanced the photosynthetic pigments, anthocyanin and protein content in rice seedlings. The results of present study has an understanding with Idrees et al. (2011) observed that foliar application of SA ameliorated the leaf chlorophyll and carotenoids content of *Catharanthus roseus* grown under saline stress. Similarly, other studies also showed the enhanced chlorophyll *a*, *b* and carotenoids content by foliar application of SA under normal and stress conditions (Yildirim et al., 2008). Jini and Joseph (2017) reported that the rice grain from high saline soil had lower protein content compared to rice grown in low saline soil, and it was increased by SA application. Protein is an important component for the rice growth and development. Chandra et al. (2007) also reported that SA application enhanced the soluble sugar and protein content in cowpea plants.

Accumulation of free radicals and oxygen species is a hallmark feature of salt induced stress which activates the antioxidant machinery of the cell. It was observed that CAT, SOD and POX enzymes activity was higher in NaCl treated plants (Fig. 3a–c) while SA application lower these enzymes activity in SA and NaCl + SA treated rice seedlings. The present study has the agreement with earlier investigations that showed the lower CAT, SOD and POX activities after SA application in salt stress (Nazar et al., 2015; Jini and Joseph, 2017). The study suggested that the application of SA ameliorated the antioxidant defense system which is critical to coping up with salt as well as other stresses.

3.2. Cell biological parameters

The rice leaves treated with control, NaCl, SA, NaCl + SA and stained with DAB to analyze the accumulation of ROS and with DAPI to detect nuclear fragmentation. The results did not show any DAB staining in control and SA treated cells. While NaCl treatment showed maximum ROS staining followed by NaCl + SA treated cells (Fig. 4). Similarly, cells stained with DAPI and showed more fragmented nuclei in NaCl treatment as compared to NaCl + SA, SA and control (Fig. 5). Control and SA treatment showed similar intact and more prominent nuclei. The foliar spray of SA on NaCl treated cells reduced the fragmentation of nuclei as compared to NaCl alone (Fig. 5).

In the present the ROS accumulation in leaves through DAB staining during salt treatment and in control leaves has been studied. DAB forms

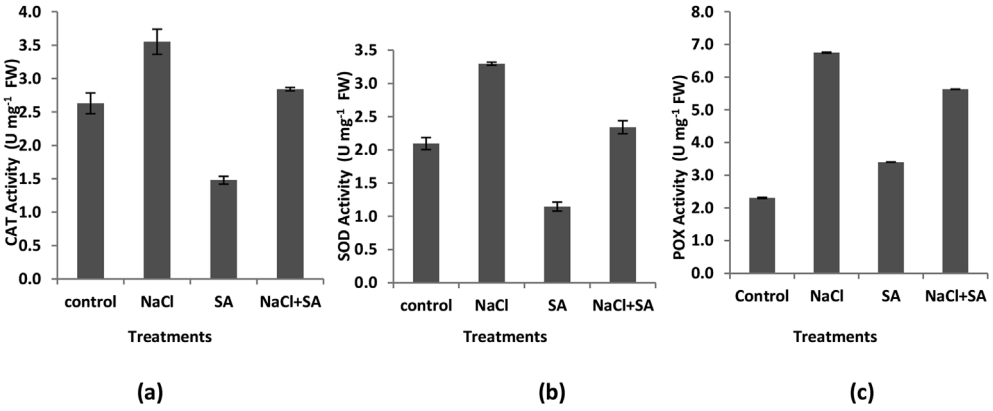

Fig. 3. Effect of NaCl, SA, NaCl + SA on (a) CAT (b) SOD (c) POX activity in *O. sativa* seedlings. The standard errors are means of three independent experiments.

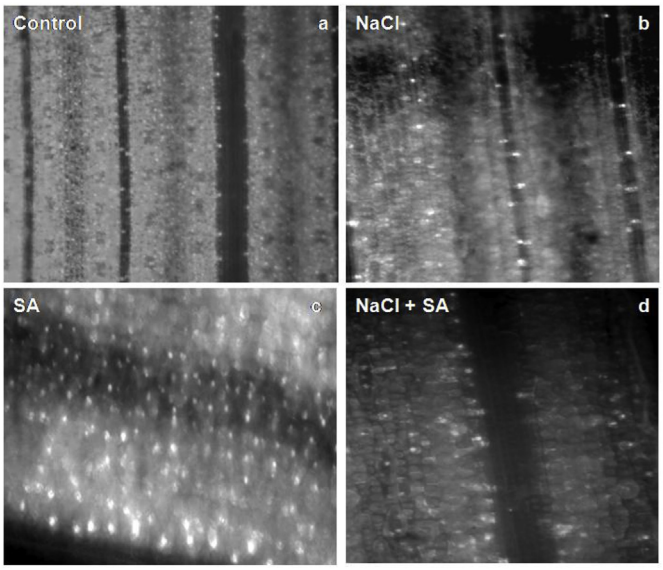

Fig. 5. Leaves of *O. sativa* (CO-51) treated with NaCl, SA, NaCl + SA and stained with DAPI to detect nuclear fragmentation under fluorescent microscope with 40X magnification.

the brown precipitate with ROS in NaCl treated leaves while application of SA reduced the ROS accumulation in leaves showed lower DAB deposition (Fig. 4). Similarly, DAPI staining showed the fragmented nuclei during salt stress in leaf cells while SA application showed intact nuclei in cells. The present study is in agreement with (Katsuhara and Kawasaki, 1996) that showed the salt stress induced the nuclear fragmentation in meristematic cells of barley root.

3.3. Gene expression analysis

The genes encoding antioxidant proteins including CAT-1, SOD and GPX, the inhibitor of the programmed cell death BI-1, MAPK-1, transcription factor WRKY53, and nine ATG genes were validated by Real-Time PCR in control, NaCl, SA, and NaCl + SA treatment. Gene expression analysis of CAT, SOD, and GPX expressed the upregulation during salt stress condition while the gene expression was reduced when SA was exogenously applied to rice seedlings (Fig. 5 a-c).

The CAT-1 enzyme involved in the elimination of H_2O_2 produced due to different stress condition and overexpressed in different plant species (Du et al., 2008; Ibrahim et al., 2018). SOD also provides tolerance to stress condition by binding with singlet oxygen. In different plant species including Arabidopsis, rice, and tomato a number of SOD genes have been analyzed during stress tolerance and showed enhanced resistance to stress condition (Kliebenstein et al., 1999). Rodriguez Milla et al. (2003) showed that GPX genes overexpressed during abiotic and biotic stresses. These gene expression blueprints agreed with earlier reports that GPX, CAT and SOD genes used different signaling pathways to different substrates (Guan et al., 2017). On the basis of antioxidant enzymes activity and gene expression study conclude that SA has strong scavenging activity and helps to remove the toxic effect of saline stress.

Mitogen-activated protein kinase (MAPKs) is a cascade of evolutionarily conserved signal transducing molecules and involved in the cellular homeostasis. The main function of these kinases is the transfer of intra and extracellular signals from receptors to effectors. The cellular responses of MAPKs include Cell division and differentiation as well as the biotic and abiotic stress response (Jagodzik et al., 2018). It is also involved in the hormonal signaling (Raja et al., 2017).

Present study showed that MAPK1 during NaCl treatment upregulated as compared to control, SA and NaCl + SA (Fig. 6b). The earlier studies on different MAPK families proved that MAPKs showed higher abiotic stress tolerance. In Arabidopsis, MKK2 overexpression showed increased salt and freezing tolerance (Teige et al., 2004) AtMEK1 overexpressed plants were more tolerant to drought and salt stresses (Xing et al., 2007). Expression of active MKK9 protein enhances the sensitivity of transgenic seedlings to salt stress, while the loss of MKK9 activity resulted in reduced salt sensitivity (Xu et al., 2008). The expression of MAPK1 downregulated in SA treatment as compared to NaCl treated rice seedlings (Fig. 6). The results of the present study showed that MAPK1 is responsible for salt-induced signaling and upregulated in high saline condition. SA provides the resistance to salt stress by maintaining the cell homeostasis through the SA signaling pathway (Yang and Guo, 2018).

Leaf senescence is a complex mechanism showed enormously altered transcriptome. It has been reported that large numbers of transcription factors get involved in the process of senescence and exhibited the up and down-regulation of genes (Zentgraf et al., 2004),. WRKY transcription factors are the second largest group of transcription factors involved in senescence (Guo et al., 2004). The function of each transcription factors is still not clear in plants although 21 WRKY TFs out of 59 were expressed during the dark-induced senescence (Lin and Wu, 2004), (Kim et al., 2018). WRYK53 is one of the important members of this family has an important role in biotic and abiotic induced senescence (Zentgraf et al., 2010; Sarkar et al., 2018). In the present study the gene expression analysis of WRKY53 showed the upregulation during NaCl treatment while the expression was downregulated after SA application (Fig. 6b). The study suggested that WRKY53 is a positive regulator of senescence and SA function as an inhibitor of salt-induced senescence in rice.

BI-1 blocks the cell death induced by Bax overexpression and function as a regulator of cell death in plants (Hernández-López et al., 2018). Present study reported that BI-1 gene showed the upregulation during the SA application in rice seedling (Fig. 6b). BI-1 gene expression was reduced in NaCl treatment but slightly enhanced in NaCl + SA treatment (Fig. 6b). The present study has an understanding with earlier reports that showed BI-1 in plants inhibited the cell death induced by H_2O_2 and SA (Ishikawa et al., 2013; Kawai-Yamada et al., 2004). The present study suggested that SA involved in the inhibition of cell death induced by salt stress and protect the plants from cellular damages.

Autophagy is a conserved process of degradation of cytoplasmic constituents to maintain the cellular homeostasis of the cell. Nutritional starvation and various abiotic and biotic stresses activated the ATG genes and expression of these genes has been proved to help in tolerance to these stresses. In the present study the expression of nine ATG genes during saline stress (Fig. 7) was analyzed. The results showed that all ATG genes were highly upregulated during the NaCl treatment as compared to SA and NaCl + SA. SA expressed the downregulation of ATG genes in SA alone and NaCl + SA treated plants. That suggested ATG genes were highly expressed in salt stress condition and SA pathway is also involved with the autophagy process and provide tolerance during saline stress or oxidative damage. Earlier (Yoshimoto et al. n. d., 2009) analyzed that autophagy negatively regulates cell death by controlling NPR1-dependent salicylic acid signaling during senescence and the innate immune response in Arabidopsis. Present study also coincides with earlier Wang et al. (2019) showed that antioxidant and autophagy genes expression was increased during drought stress in peach leaves. The study suggested that plant autophagy operates a novel negative feedback loop modulating SA signaling to negatively regulate senescence and immunity-related PCD. In another study (Du et al., 2008; Wang et al., 2016) reported that salicylic acid promotes autophagy via NPR3 and NPR4 in *Arabidopsis* senescence and innate immune response. The present study also suggested that SA and autophagy pathway are interconnected and co-regulated during saline stress and enhance the salt tolerance in rice seedlings.

4. Conclusion

SA is an important signaling molecule and plays important in plant

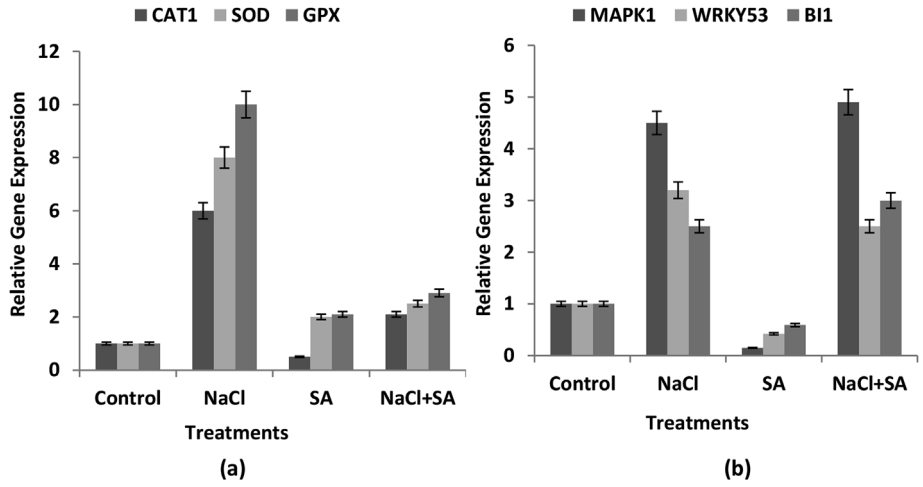

Fig. 6. Effect of NaCl, SA, NaCl + SA on (a) CAT1, MnSOD, GPX (b) MAPK1, WRKY53, BI1 genes expression in *O. sativa*. The data shown are $2^{-\Delta\Delta CT}$ expression ratio of target gene with normalization to Actin as an internal control.

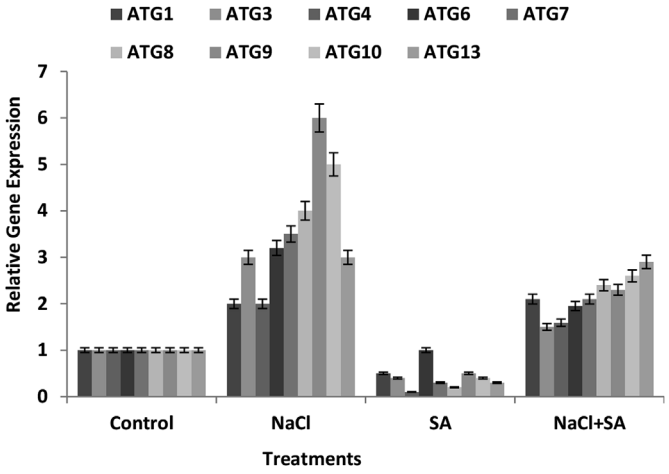

Fig. 7. Effect of NaCl, SA, NaCl + SA on ATG expression in *O. sativa*. The data shown are $2^{-\Delta\Delta CT}$ expression ratio of target gene with normalization to Actin-1 as an internal control.

defense responses against various environmental stimuli. SA pathway is not an isolated pathway although it is an interconnected network of various well-regulated pathways. Present study attempted to analyze the role of SA during salt stress at the biochemical, cellular and molecular level. Study concluded that all physiological, biochemical, cellular and molecular parameters were significantly reduced under the applied salinity level (100 mM NaCl). The given saline stress was designed as a method to more rapidly and visibly help to identify salt sensitive plants. The 100 mM NaCl not only showed the measurable phenotypic parameters during short period but regardless of saline stress all plants was also able to recover after treatment and survived. High salinity enhanced the ROS accumulation in cells, induces the cell death like features such as nuclear fragmentation in rice leaf cells, enhanced antioxidant enzymes activity. SA application revealed that it does not only alleviate the effect of salt stress although it also induces various genes including CAT, SOD, GPX, MAPK1, WRKY53, BI-1 and ATG. The application of SA at 0.5 mM shows the promotive effect and enhanced the tolerance in saline stressed rice cultivar. This showed the advantage over other higher concentration that may cause toxicity. Study also confirmed the involvement of SA, autophagy and cell death pathways during saline stress in rice and suggested that SA pathway involved with regulation of autophagy and PCD mechanism and critical for salt tolerance in rice.

Competing interest

Authors declares no competing interest.

Authors contribution

MSK and TA carried out the experimental work recorded the data and wrote the manuscript. SH supervised all the experiments and verified the results. SH and MADB reviewed and proofread the manuscript. All authors have read and approved the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101027.

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