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Involvement of poly(ADP-ribose)ylation (PARylation) in the regulation of antioxidant defense system in *Arabidopsis thaliana* under salt stress

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Abstract: Posttranslational modifications (PTM) are one of the first responses of plants to environmental stress and involve changing the location and activity of proteins in the cell. Addition of poly(ADP-ribose) (PAR) to proteins, poly(ADP-ribose)ylation (PARylation), is a posttranslational modification resulting from the binding of ADP-ribose from NAD^+ to target proteins by PAR polymerases (PARP). PARylation is involved in many physiological events in plants including abiotic and biotic stress response. The aim of this work was to understand involvement of PARylation in inducing enzymatic antioxidant defence and alternative electron sinks in response to salinity. For this purpose *A. thaliana* plants were treated with 3-aminobenzamide (3-AB), which is a PARP inhibitor, in the presence of 100 mM NaCl. The 3-AB treatment induced plant fresh weight under control and salinity conditions. Moreover, 100 mM NaCl + 3-AB treated plants had lower lipid peroxidation when compared to 100 mM NaCl group indicating mitigation of oxidative stress. This oxidative stress mitigation was achieved by significantly induced superoxide dismutase (SOD) activity and transcriptional activation of genes related to ROS scavenging such as *MSD1*, *CAT1*, *APX1*, *GR1*. On the other hand, transcriptional regulation of mitochondrial alternative oxidase (AOX) pathway was also induced with 3-AB treatment (*AOX1a* and *AOX1d*) under salt stress indicating that ROS avoidance mechanisms are also activated along with ROS scavenging. However, in contrast to AOX, chloroplastic plastid terminal oxidase pathway was not induced with 3-AB.

Keywords: 3-aminobenzidine, antioxidant enzymes, *Arabidopsis thaliana*, poly(ADP-ribose)ylation, reactive oxygen species, salt stress

1. Introduction

Soil salinity is a major and widespread environmental factor that negatively affects plant growth and productivity in 20% of arable land (FAO, 2000). The detrimental effects of salinity on plants occur mainly in two ways. First, decreased soil water potential due to increased solute levels leads to osmotic stress, which makes it difficult for a plant to extract water from the soil mixture. Second, at later stages of stress, excessive accumulation of ions in leaf cells causes ion toxicity, hence the ionic effect. In addition, salinity can reduce the uptake of ions such as K^+ and Ca^{+2}, causing a loss of ion balance in the plant cells (Munns and Tester, 2008). These detrimental effects of salinity disrupt energy metabolism in mitochondria and chloroplasts of plants. As a result, reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide anion ($O_2\bullet^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH•) accumulate excessively in the cell. ROS are produced under normal conditions in all aerobic organisms including plants as inevitable byproducts of aerobic metabolism (Bose et al., 2014). ROS production in the plant cell takes place intensively in peroxisomes, chloroplasts, mitochondria, cell membrane and apoplast. It is now well documented that ROS have a dual role as ROS can act as secondary messengers in cellular signalling pathways at low levels. On the other hand, ROS are toxic when they accumulate at high levels due to environmental stresses such as salt stress. Therefore, ROS should be kept at a low level in the cell to perform their signalling function, and this is achieved by keeping the ROS production and detoxification mechanisms in the cell in a delicate balance, which involves enzymatic and nonenzymatic antioxidants spread within cellular compartments (Mittler et al., 2004). Moreover, uncontrolled and excessive accumulation of ROS can cause damage to biomolecules by oxidizing proteins, lipids and nucleic acids (Noctor and Foyer, 1998).

Plant adaptation to salinity involves regulatory mechanisms at different levels such as transcriptional, posttranscriptional, translational and posttranslational levels (Wu et al., 2021). The addition of poly(ADP-ribose) (PAR) derived from nicotinamide adenine dinucleotide (NAD^+) to lysine residues of target proteins

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is called poly(ADP-ribose)ylation (PARylation). This modification is catalysed by PAR polymerases (PARPs). PARPs, exclusively found in eukaryotes, were first identified in plants during the mid-1990s (Poltronieri, 2020; Groslambert et al., 2021). Utilization of NAD^+ as a substrate, hence the conversion of NAD+ to nicotinamide, links PARylation to energy homeostasis of the cell and eventually to programmed cell death (PCD) (Taipakova et al., 2020). Arabidopsis encodes three PARP genes (PARP1-3), which have been implicated in tolerance to abiotic and biotic stress responses (Briggs and Bent, 2011) besides regulation of developmental processes (Rissel and Peiter, 2019), DNA repair (Blue et al., 2021; Mitra and Dey, 2021) and transcriptional regulation (Vainonen et al., 2021). Inhibition of PARP activity employing gene knockout or chemical inhibition confers tolerance to various abiotic stresses such as high temperature, high light and drought (De Block et al., 2005; Vanderauwera et al., 2007; Schulz et al., 2012) which is correlated with decreased levels of poly(ADP-ribose).

Different chemicals that can inhibit PARP activity are used to study the role of PARPs and since these inhibitors target the PARP catalytic site, all proteins with PARP activity can be inhibited (Rouleau et al., 2010). 3-aminobenzamide (3-AB), nicotinamide, and 3-methoxybenzamide (3-MB) are inhibitors that are widely used in animal systems and they inactivate PARP enzymes by mimicking NAD^+ structure (Klaidman et al., 2003; Koh et al., 2005; Makogon et al., 2010). These inhibitors have also been shown to be effective in plant systems (Chen et al., 1994; Berglund et al., 2017; Rissel et al., 2017; Chetty et al., 2020).

In animals, it has been reported that chemicals that inhibit PARylation, such as 3-AB, stimulate the antioxidant defence system, and they especially regulate superoxide dismutase (SOD) and catalase (CAT) activities and the levels of glutathione (Sriram et al., 2015). However, studies on the role of PARylation in redox regulation in plants are limited. It has been demonstrated that PARP inhibitors (3-AB and nicotinamide) reduced the degree of cell death triggered by H_2O_2 in cultured soybean cells (Amor et al., 1998). In another study, PARP inhibition provided tolerance to salt stress in *A. thaliana* and plants had a higher level of NAD^+, while reduced ascorbate and glutathione levels remained the same (Schulz et al., 2012). However, the effects of PARylation on enzymatic components of antioxidant defence remain unknown. Since PARylation affects NAD^+ levels, plant cells have to adapt to a new redox situation, which often involves regulation of enzymatic antioxidants. Therefore, the current study aims to elucidate how the antioxidant defence system of plant cells is regulated in response to inhibition of PARylation under salt stress. For this purpose, PARylation was inhibited by the application of 3-AB to the model plant *A. thaliana*. Afterward, the responses of plants with and without inhibited PARylation to salt stress were determined by (i) determination of growth and lipid peroxidation, (ii) elucidating changes in PAR metabolism with expressions of *PARP1*, *PARP2*, *PARG1* and *PARG2*, (iii) measuring the activities of enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX) and glutathione reductase (GR), (iv) determining expressions of antioxidant enzyme encoding genes such as *MSD1* (*MnSOD1*), *FSD1 (FeSOD1), CSD1 (CuZnSOD1), CAT1, APX1* and *GR1*, (v) determining expressions of genes involved in alternative electron pathways in chloroplasts (plastid terminal oxidase = *PTOX*) and mitochondria (alternative oxidase = *AOX1a* and *AOX1d*).

2. Material and methods

2.1. Plant materials and treatments

Arabidopsis thaliana Col-0 ecotype was used during experiments. The seeds were kept in 70% ethanol for 1 min and washed 5 times with sterile distilled water. Following this, seeds were sterilized in 4% NaOCl (sodium hypochlorite) solution for 10 min and washed 5 times again with sterile distilled water. Seeds were stratified at 4 °C for 72 h to synchronize germination.

Plants were germinated and grown in Petri dishes containing 1/2 strength Murashige and Skoog (MS) medium containing 1% sucrose and 0.8% agar (Gamborg et al., 1968). Following germination for 2 days, plants were grown in a plant growth room (150 µmol m^{-2} s^{-1} light intensity (12 h light/12 h dark), 22 °C temperature and 60% relative humidity) in MS medium for 4 days and these plants were used in further experiments.

For inhibition of PARP activity, hence PARylation, 0.75 mM 3-aminobenzamide (3-AB) was used according to Zhang et al. (2015). 3-AB was mixed into sterilised MS medium after it cooled to 60–70 °C. Salt stress was imposed by addition of 100 mM NaCl to the MS media. Plants were treated with 3-AB, 100 mM NaCl or 100 mM NaCl + 3-AB for 7 days, MS media with no additions served as control. Combined treatments of NaCl and 3-AB were done simultaneously. At the end of 7 days of treatments, samples were frozen in liquid nitrogen and stored at –80 °C until analysis.

2.2. Measurement of physiological parameters

Fresh weights of at least 6 seedlings for each treatment group were recorded after 7 days of treatments.

2.3. Lipid peroxidation

Lipid peroxidation was measured by determining the TBARS (thiobarbituric acid reactive substances) levels according to Rao and Sresty (2000). 0.1 g of sample (pooled whole seedlings) was homogenized with 0.1%

trichloroacetic acid (TCA) and was centrifuged at 10,000 g for 5 min at 4 °C. The supernatant was mixed with reaction mixture (20% TCA, 0.5% TBA) and incubated at 95 °C for 1 h. TBARS concentration was calculated based on absorbance at 532 nm. The nonspecific turbidity was measured at 600 nm and subtracted from the absorbance at 532 nm. While calculating total TBARS concentration, extinction coefficient of 155 mM^{-1} cm^{-1} was used.

2.4. Quantitative RT-PCR analysis

Total RNA was isolated from 0.1 g of fresh samples using Plant Total RNA Isolation kit (Norgen) according to the directions of the manufacturer. DNA was digested with DNAse I to remove any residual genomic DNA. Reverse transcription was performed using Transcriptor First Strand cDNA synthesis kit (Roche) with 1 µg of total RNA. The resulting cDNAs were used as templates for qRT-PCR. PCR program was as follows: 95 °C for 5 min, 40 cycles at 95 °C for 15 s, 15 s at 60 °C and 30 s at 72 °C. SYBR Green I Master Mix (Roche) was used for qRT-PCR reactions. Results were analysed with StepOne Plus software (Applied Biosystems). Control group (without salt and 3-AB) was selected as the reference and its value was set to 1 when calculating the expression levels of genes of interest. *ACTIN8* was used as the housekeeping gene and expressions of other genes were normalized according to it (Wu et al., 2012). Primers used during qRT-PCR are given in Table 1.

2.5. Determination of antioxidant enzyme activities

All steps for protein extraction were carried out at 4 °C to keep protease activity at a minimum. Enzyme

Table 1. List of primers used for qRT-PCR analysis.

Gene no	Primer name	Oligo sequence (5’-3’)
AT3G22370	AOX1a-F	CTGGACCACGTTTGTTC
	AOX1a-R	ACACCCCAATAGCTCG
AT1G32350	AOX1d-F	TACCGCACTCTTCGAC
	AOX1d-R	GGCTGGTTATTCCCACT
AT4G22260	PTOX-F	CCAGTGGAGAGGAGTTGAAGA
	PTOX-R	AGATTTTCTATTACTGGTCTTCGAG
AT4G02390	PARP1-F	ATGCTACTCTGGCACGGTTCAC
	PARP1-R	AGGAGGAGCTATTCGCAGACCTTG
AT2G31320	PARP2-F	ATCGTCTACGATACAGCCCAGGTG
	PARP2-R	TGGTTCAGGCTCATCTCTTGTGC
AT2G31870	PARG1-F	CGGATGGATGACAATGAAGCT
	PARG1-R	ATGTACTCACCAGCAAACCGAAA
AT2G31865	PARG2-F	TTTGTTTCTTATCCCAAGGCTGAT
	PARG2-R	CTTCTATAGCTCCCGAGGTGTGA
AT3G10920	MSD1-F	ATGTTTGGGAGCACGCCTAC
	MSD1-R	AACCTCGCTTGCATATTTCCA
AT4G25100	FSD1-F	CTCCCAATGCTGTGAATCCC
	FSD1-R	TGGTCTTCGGTTCTGGAAGTC
AT1G08830	CSD1-F	TCCATGCAGACCCTGATGAC
	CSD1-R	CCTGGAGACCAATGATGCC
AT1G20630	CAT1-F	AAGTGCTTCATCGGGAAGGA
	CAT1-R	CTTCAACAAAACGCTTCACGA
AT1G07890	APX1-F	TGCCACAAGGATAGGTCTGG
	APX1-R	CCTTCCTTCTCTCCGCTCAA
AT3G24170	GR1-F	TGCTGCTAGGTTTTCGGCTAAT
	GR1-R	CCTCAGAGCTAATAGGGTGAAATGG
AT1G49240	ACT8-F	TCAGCACTTTCCAGCAGATG
	ACT8-R	ATGCCTGGACCTGCTTCAT

extracts were prepared by grinding 0.1 g of samples (pooled whole seedlings) to a fine powder in liquid nitrogen followed by homogenization with 0.5 mL extraction buffer (50 mM sodium phosphate pH 7.8, 1 mM ethylenediaminetetraacetic acid (EDTA), 2% (w/v) polyvinylpolypyrrolidone (PVPP)). For ascorbate peroxidase activity analysis, 5 mM ascorbic acid was added to the homogenization buffer. After homogenization, the samples were centrifuged at 10,000 g for 5 min (Uzilday et al., 2017). The supernatants obtained were used to determine the protein amount and enzyme activity. Bovine serum albumin (BSA) was used as a standard and the amount of protein was detected according to Bradford (1976).

For determination of superoxide dismutase (SOD (EC 1.15.1.1)) activity inhibition of photochemical reduction of NBT by SOD at 532 nm was observed (Beauchamp and Fridovich, 1971). Catalase (CAT (EC 1.11.1.6)) activity was determined by monitoring the scavenging of H_2O_2 at 240 nm (Bergmeyer,1970). The amount of catalase that scavenges 1 µmol H_2O_2 per minute was calculated as 1 U. Peroxidase (POX (EC 1.1.11.1.7)) activity was measured according to the method used by Herzog and Fahimi (1973) by monitoring oxidation of 3,3'-diaminobenzidine (DAB) at 465 nm. Ascorbate peroxidase (APX (EC 1.11.1.11)) activity was measured according to Nakano and Asada (1981). The oxidation of ascorbate by APX was monitored at 290 nm and the amount of oxidized ascorbate was calculated using the extinction coefficient of 2.8 mM^{-1} cm^{-1}. The amount of APX that oxidizes 1 µmol of ascorbate per minute was calculated as 1 U. GR activity was measured according to Foyer and Halliwell (1976). NADPH oxidation was monitored at 340 nm. The amount of oxidized NADPH was determined using the extinction coefficient 6.2 mM^{-1} cm^{-1}. The amount of GR that oxidizes 1 µmol of NADPH per minute was calculated as 1 U.

2.6. Isoenzyme determination of antioxidant enzymes

Proteins were separated by nondenatured polyacrylamide gel electrophoresis (Native-PAGE) and stained with enzyme-specific dye solutions. The homogenization procedure used in the activity analysis was used in the preparation of the samples. Equal amounts of protein (75 µg) were loaded into each well. Native-PAGE was done without using SDS according to Laemmli (1970). For determination of SOD isoenzymes, proteins were separated with a 12.5% gel and SOD activity was stained with riboflavin and nitrobluetetrazolium (NBT) dye according to Beauchamp and Fridovich (1971). CAT isoenzymes were separated with a 7.5% gel and bands were stained according to Woodbury et al. (1971). GR isoenzymes were seperated using 7.5% separation gel and stained according to Hou et al. (2004). Gels were visualized on the Vilbert Lourmat gel imaging system and band intensities were analysed using Bio-Profil Bio-1D software.

2.7. Statistical Analysis

Experiments were repeated 3 times and with at least two technical replicates (n = 6). The obtained data were evaluated with one-way ANOVA analysis and TUKEY post-test was applied to determine the statistical difference between different treatments. As a result of this comparison, comparisons with $p < 0.05$ were evaluated as statistically different from each other. Statistical analyses were performed with GraphPad software.

3. Results

Only 3-AB treatment enhanced fresh weight of plants by 20% while salinity decreased it by 39% as compared to control. On the other hand, 3-AB treatment under salinity decreased the FW by 26% as compared to control. This indicates that inhibition of PARylation under salinity leads to better plant performance when compared to only salt-treated plants (Figure 1A).

Only 3-AB alone did not cause a significant change in TBARS content while only salinity enhanced it by 53% whereas an increase of 33% was observed with NaCl+ 3-AB treatment (Figure 1B). Since TBARS content is a significant marker of a cellular oxidative damage, these results indicate that inhibition of PARylation prevents oxidative damage.

The expression of *PARP1* decreased by salinity and NaCl + 3-AB treatment by 1.9-fold and 2.2-fold as compared to control levels while expression of *PARP2* was enhanced by 4.6-fold and 6.8-fold in the same groups as compared to controls, respectively (Figures 2A and 2B). On the other hand, the expression of *PARG1* was enhanced with salinity by 5.5-fold and NaCl + 3-AB induced it by 7.4-fold. The expression of *PARG2* also increased with salinity by 5.5-fold and NaCl + 3-AB induced it by 9-fold (Figures 2C and 2D).

SOD activity was enhanced by salinity and NaCl + 3-AB treatments and 4 different SOD isoenzymes were determined (Figure 3A). Of these isoenzymes, 1 was characterized as MnSOD, 2 as FeSOD (1 and 2) and the last as CuZnSOD. MnSOD activity increased with NaCl treatment as compared to control (Figure 3B). On the other hand, CuZnSOD isoenzyme decreased with 3-AB treatment as compared to control. With the combination of NaCl + 3-AB, an increase in CuZnSOD activity was determined as compared to the control. FeSOD isoenzyme activity increased in the NaCl + 3-AB treatment.

The expression of *MSD1* gene did not change significantly with 3-AB and only salinity (Figure 3C). Interestingly, NaCl + 3-AB enhanced the expression of *MSD1* by 3.8-fold as compared to control levels. The expression of *CSD1* gene was enhanced by 5.3-fold under salinity while NaCl + 3-AB enhanced it by

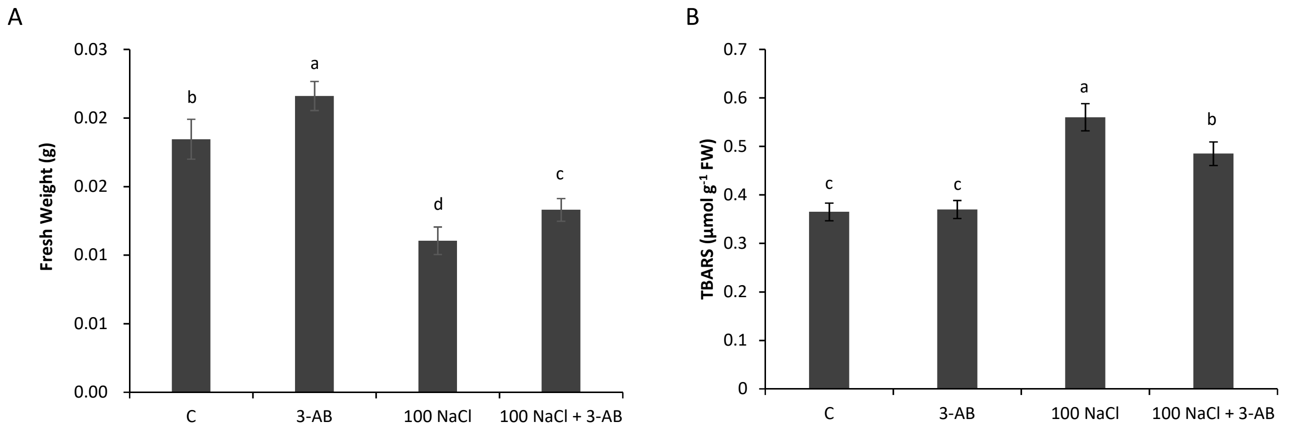

Figure 1. Evaluation of the response of *A. thaliana* plants to 3-AB under salinity. A) Fresh weight, B) lipid peroxidation (TBARS) content of *A. thaliana* treated with 3-AB and with or without 100 mM NaCl. The Bars = SE.

Figure 2. The response of PARylation related genes to 3-AB under salinity. The expressions of *PARP1, PARP2, PARG1* and *PARG2* genes of *A. thaliana* plants treated with 3-AB and with or without 100 mM NaCl. The Bars = SE.

7-fold as compared to control. On the other hand, only 3-AB treatment induced *CSD1* expression by 1.8-fold. Treatments used in this work did not affect the expression of *FSD1* significantly.

One CAT isoenzyme (a mixture of homo- and heterotetramers) was determined with native-PAGE analysis and no new band occurred with NaCl or 3-AB treatments (Figure 4A). CAT activity increased by 12% with only 3-AB application, while salinity treatment increased CAT activity by 19% compared to the control (Figure 4B). On the other hand NaCl + 3-AB combination enhanced it by 11%. The expression of *CAT1* gene was enhanced with

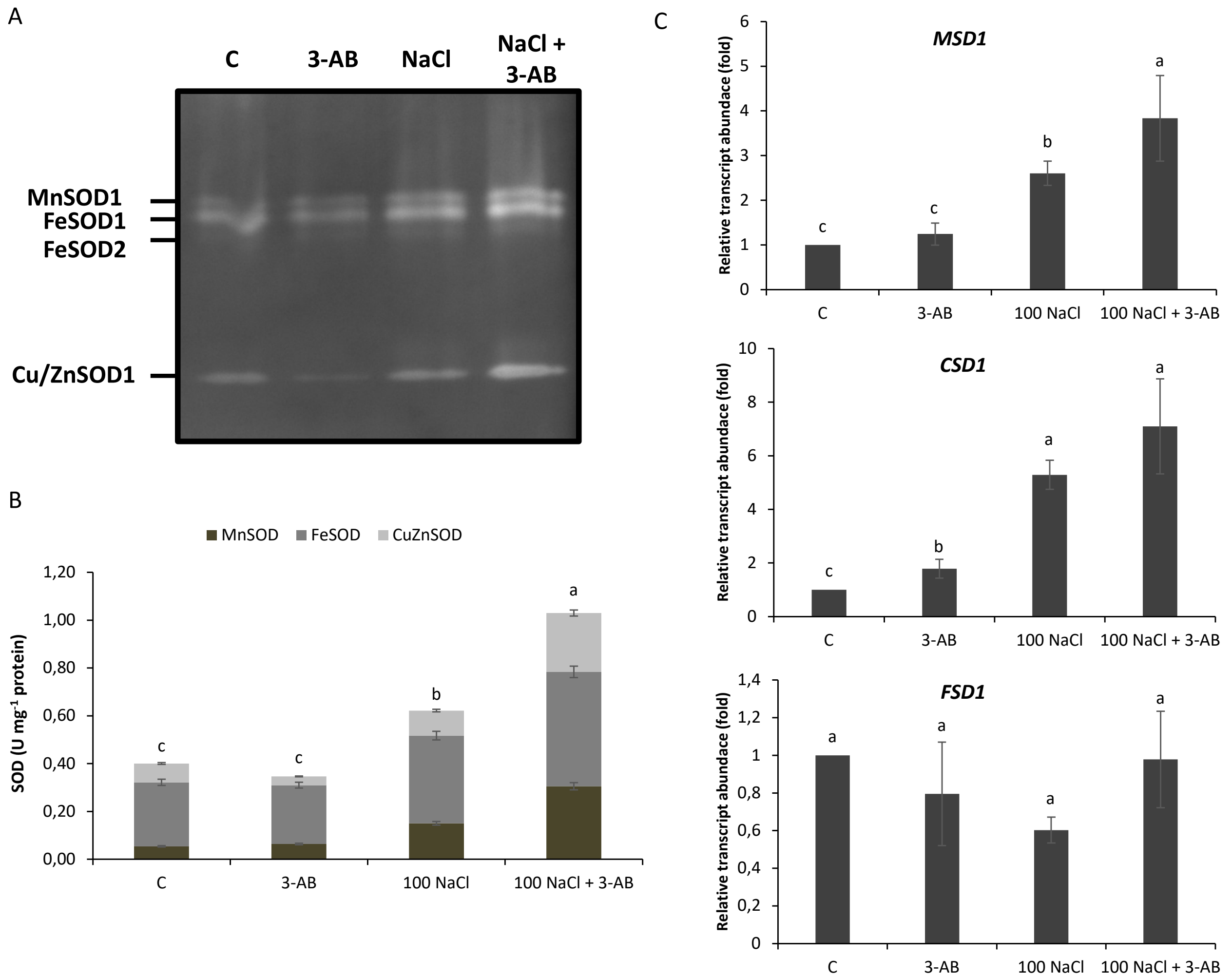

Figure 3. The response of superoxide dismutase enzyme to 3-AB under salinity. A) Native-PAGE separation of SOD isoenzymes, B) the activity of SOD enzyme, C) the expressions of MSD1, CSD1 and FeSD1 genes of *A. thaliana* plants treated with 3-AB and with or without 100 mM NaCl. The Bars = SE.

NaCl treatment by 4.8-fold while NaCl + 3-AB increased the expression of *CAT1* by 9-fold as compared to control (Figure 4C).

The activity of APX remained stable with only 3-AB treatment. On the other hand, salt stress enhanced it by 34%. Similarly, NaCl+ 3AB combination induced it by 36% as compared to controls (Figure 5A). The expression of *APX1* did not change significantly with 3-AB treatment. *APX1* expression was induced by 1.6-fold with salinity; however, NaCl + 3-AB combination resulted in 2.3-fold increase as compared to control levels (Figure 5B).

The activity of POX did not change with 3-AB treatment significantly, while salinity enhanced it by 61%. On the other hand, NaCl + 3-AB treatment induced POX activity by 51% as compared to controls (Figure 5C).

In the current study, two GR isoenzymes were determined. GR1 and GR2 isoenzyme activities increased with 3-AB treatment (Figure 6A). Only 3-AB treatment did not have any effect on GR activity (Figure 6B). On the other hand, salinity and NaCl + 3-AB treatment induced GR activity by 32% and 20%, respectively. The expression of *GR1* was detected as higher than that of control levels under only salinity and NaCl + 3-AB treatments by 5-fold and 8.8-fold, respectively (Figure 6C).

The expressions of alternative electron transport pathway elements were also determined to evaluate the effects of PARylation on mechanisms that relax electron transfer chains in chloroplasts and mitochondria. The expression of *AOX1a* was enhanced by 6.4-fold with salinity. On the other hand, NaCl + 3-AB treatment enhanced it by 11-fold as compared to controls (Figure 7A). The expression of *AOX1d* also showed a similar pattern, only salinity enhanced it by 6.4-fold and NaCl + 3-AB increased it by-8.6 fold (Figure 7B). The expression of *PTOX* did not show any statistically significant differences under these conditions (Figure 7C).

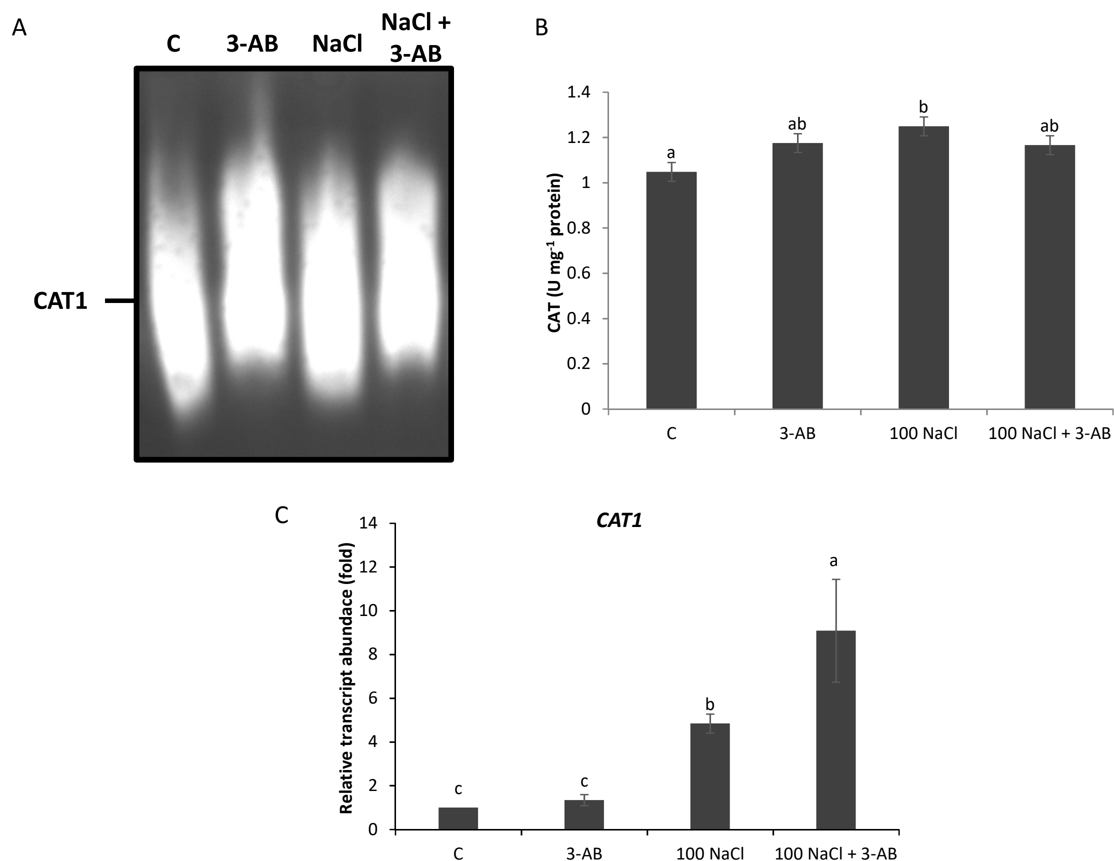

Figure 4. The response of catalase enzyme to 3-AB under salinity. A) Native-PAGE separation of CAT isoenzymes, B) the activity of CAT enzyme, C) the expression of *CAT1* gene of *A. thaliana* plants treated with 3-AB and with or without 100 mM NaCl. The Bars = SE.

4. Discussion

The use of chemical inhibitors is advantageous to overcome genetic redundancy such as in the case of PAR metabolism. Although treatment of plants with molecules such as ABA, salicylic acid, H_2O_2 or NO to increase plant stress tolerance has been widespread in the literature (Fujita et al., 2005; Janda et al., 2007; Mirza et al., 2010; Li et al., 2011; Hossain et al., 2015; Khan et al., 2015; Ryu and Cho, 2015; Hasanuzzaman et al., 2018), utilization of molecules that inhibit specific enzymes or pathways to increase plant stress tolerance are limited.

In this work, the chemical inhibition of PARP increased plant growth even under nonsaline control conditions. Moreover, 3-AB treated plants had higher FW under salt stress. Previously, similar results have also been obtained for high light and high temperature stresses (Schulz et al., 2012). Although lipid peroxidation levels did not change with 3-AB treatment under control conditions, 3-AB treatment decreased lipid peroxide levels under salt stress. This demonstrates that the chemical inhibition of PARP activity results in more efficient protection against salt-induced oxidative stress. Generally there is a trade-off between abiotic stress tolerance resistance and growth under normal conditions for molecules that induce abiotic stress tolerance (Bechtold and Field, 2018). However, this seems not to be the case for PARP inhibition, which can induce growth under normal conditions and mitigate salt stress at the same time under stressed conditions. Results obtained in the current work in regards to plant performance indicate that manipulation of PAR metabolism via biotechnological tools or chemical treatment with PARP inhibitors has the potential to increase plant performance and abiotic stress tolerance of plants.

For further exploring the effects of chemical PARP inhibition on antioxidant defence, activities of key antioxidant enzymes and their transcriptional response were determined. SODs are vital enzymes that can convert $O_2^{\cdot-}$ to H_2O_2 and are named as the first line of defence against ROS (Foyer, 2018). $O_2^{\cdot-}$ can be produced in chloroplasts by Mehler reaction via PSI or in mitochondria by complexes I, II and IV. Moreover, salinity disrupts gas exchange via closing stomata, hence impairing balance between light reactions and the Calvin cycle resulting

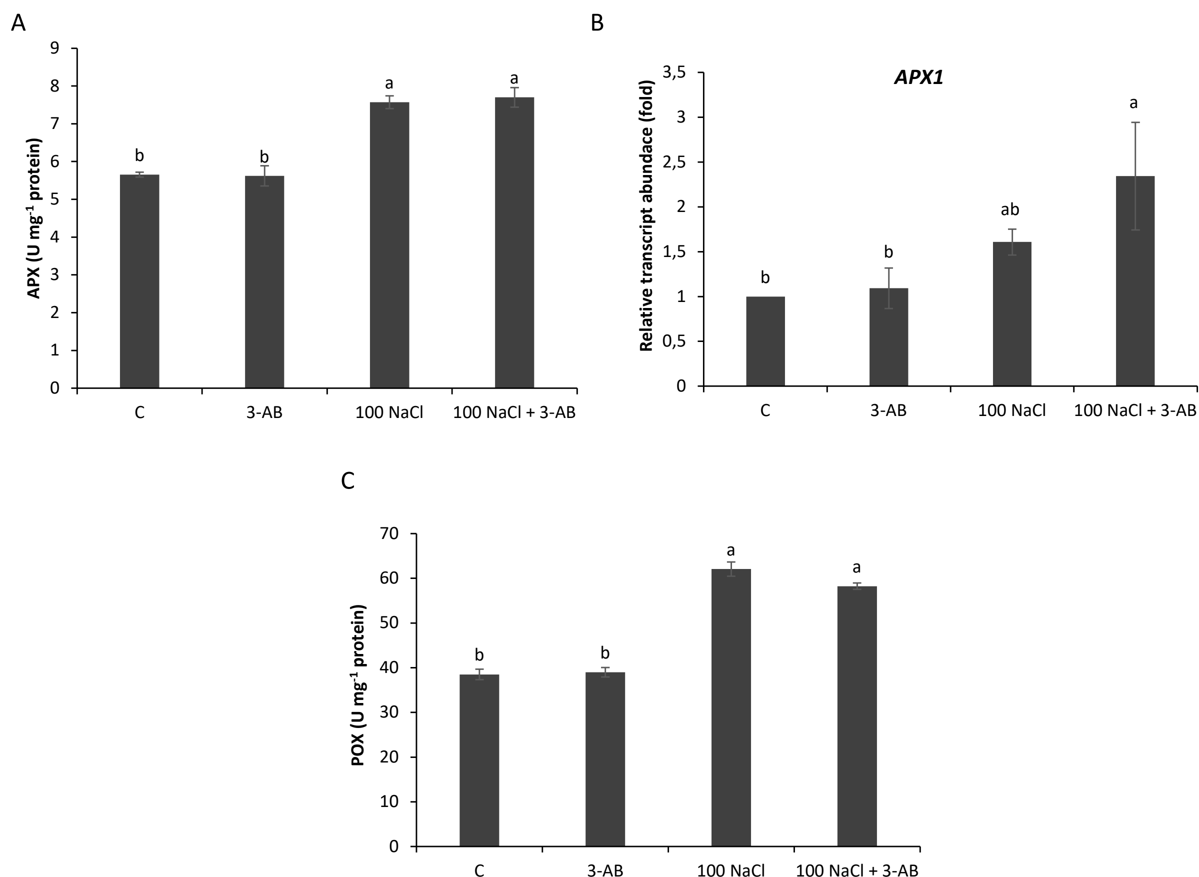

Figure 5. The response of ascorbate peroxidase and peroxidase enzymes to 3-AB under salinity. A) The activity of APX enzyme, B) the activity of POX enzyme, C) the expression of *APX1* gene of *A. thaliana* plants treated with 3-AB and with or without 100 mM NaCl. The Bars = SE.

in increased $O_2^{\bullet -}$ production (Foyer and Noctor, 2003). Limiting diffusion of CO_2 into the leaf also enhances the rate of photorespiration in C_3 plants such as *A. thaliana*, which leads to increased H_2O_2 production in peroxisomes and also $O_2^{\cdot -}$ in mitochondria due to increased electron flow through mitochondrial electron transfer chain to regenerate NADH produced by photorespiration (Lim et al., 2020). In the current study, salinity induced the activity of SOD as reported previously (Tsugane et al., 1999; Wang et al., 2004); however, NaCl + 3-AB treated plants had higher SOD activity, which resulted in better protection against $O_2^{\bullet -}$. All three type of SOD (FeSOD, MnSOD and CuZnSOD) was induced as evident by native-PAGE gel. This indicates a general induction in SOD activity rather than an induction specific to a compartment of the cell since FeSOD is generally found in chloroplasts, while MnSOD is localized in mitochondria (Gomez et al., 2004). However, among the genes encoding SOD, only *MSD1* showed a significant difference between salinity and NaCl + 3-AB treatment. Although there are no studies that investigate relationship between PARylation and antioxidant enzymes in plants, in animals it has been demonstrated that overexpression of MnSOD in mitochondria can protect cells against PARP mediated cell death and there is a reciprocal relationship between PARP and MnSOD (Kiningham et al., 1999).

CAT is mainly responsible for the scavenging of H_2O_2 in the peroxisomes that is generated via photorespiration (Su et al., 2018). Although NaCl treatment increased CAT activity significantly, it returned to control levels with 3-AB treatment under salt stress. However, 3-AB treatment both under saline and nonsaline conditions shifted CAT isoenzyme pattern. CAT forms homo- or heterotetramers and due to this it is difficult to obtain separate bands on native-PAGE gels (Williamson and Scandalios, 1993). Nevertheless, it can be seen that CAT band obtained in 3-AB treated groups (only 3-AB and NaCl + 3-AB) shift up in the gels indicating a change in the profile of CAT homo- or heterotetramers. More interestingly, NaCl + 3-AB treatment induced *CAT1* transcription significantly although this was not reflected in the total CAT activity. This induction of *CAT1* expression which might have a role in regulation of CAT homo- or heterotetramers profile.

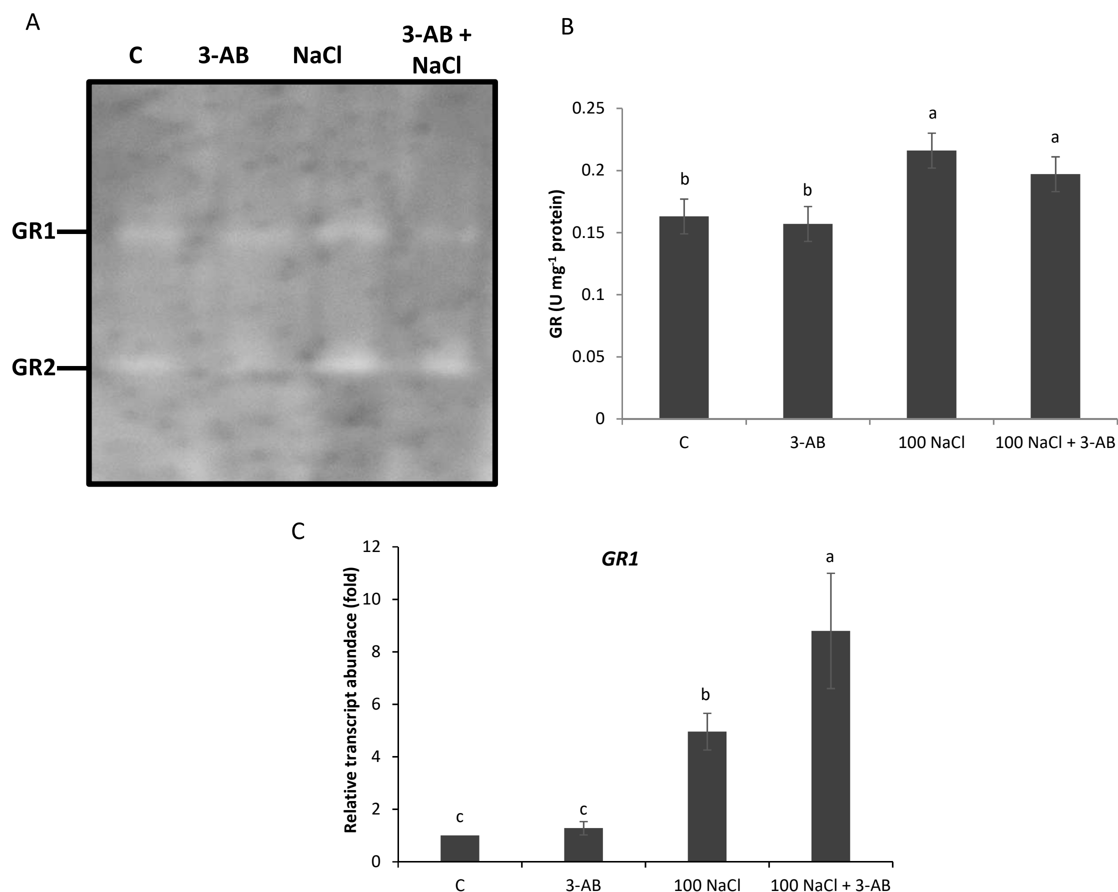

Figure 6. The response of glutathione reductase enzyme to 3-AB under salinity. A) Native-PAGE separation of GR isoenzymes, B) the activity of GR enzyme, C) the expression of *GR1* gene of *A. thaliana* plants treated with 3-AB and with or without 100 mM NaCl. The Bars = SE.

Aside from CAT, APX and POX are responsible for scavenging of H_2O_2 in various compartments of the plant cell (Caverzan et al., 2012). However, catalytic properties and subcellular localizations of APX and POX are different when compared to CAT. For example, APX has a higher affinity for H_2O_2 but lower catalytic rate (De Gara et al., 1997). Moreover, besides other compartments, APX is localized in chloroplasts, which makes it a major component against chloroplastic ROS (Asada, 1992). Salt stress induced APX and POX activity, but in contrast to SOD, inhibition of PARylation during salt stress with 3-AB did not have additional effect on activities of these enzymes. These findings indicate that induction of H_2O_2 scavenging enzymes such as CAT, APX or POX might not directly contribute to oxidative stress tolerance conferred by 3-AB under salinity.

Moreover, besides providing a snapshot of ROS scavenging enzymes, current study also aims to elucidate changes in mechanisms that avoid ROS production in the first place. Chloroplast and mitochondria produce ROS when electron transfer chain components are overreduced and cannot accept new electrons (Foyer and Noctor, 2003). Flow of electrons to O_2 instead of electron transfer chain components results in ROS formation. On the other hand, excitation energy transfer at PSII can lead to $^1O_2$ production (Foyer, 2018). Therefore, it is of paramount importance to relax electron pools in chloroplast and mitochondria to avoid excess ROS production. For this purpose, two very similar proteins, alternative oxidase (AOX) in mitochondria and plastid terminal oxidase (PTOX) in chloroplasts are utilized by plants (McDonald and Vanlerberghe, 2006). Alternative oxidase oxidizes ubiquinone pool in mitochondria, while plastid terminal oxidase, also named IMMUTANS, oxidizes plastoquinone pool in chloroplast (Aluru and Rodermel, 2004). By doing so, AOX relax electron load on complex I and PTOX relax electron and excitation energy load on PSII. This relaxation prevents ROS production and dissipates energy from electron transfer chains (Arnholdt-Schmitt et al., 2006). Interestingly, results of the current study implicate that 3-AB treatment induces *AOX1a* and *AOX1d*. Among AOX encoding genes (total

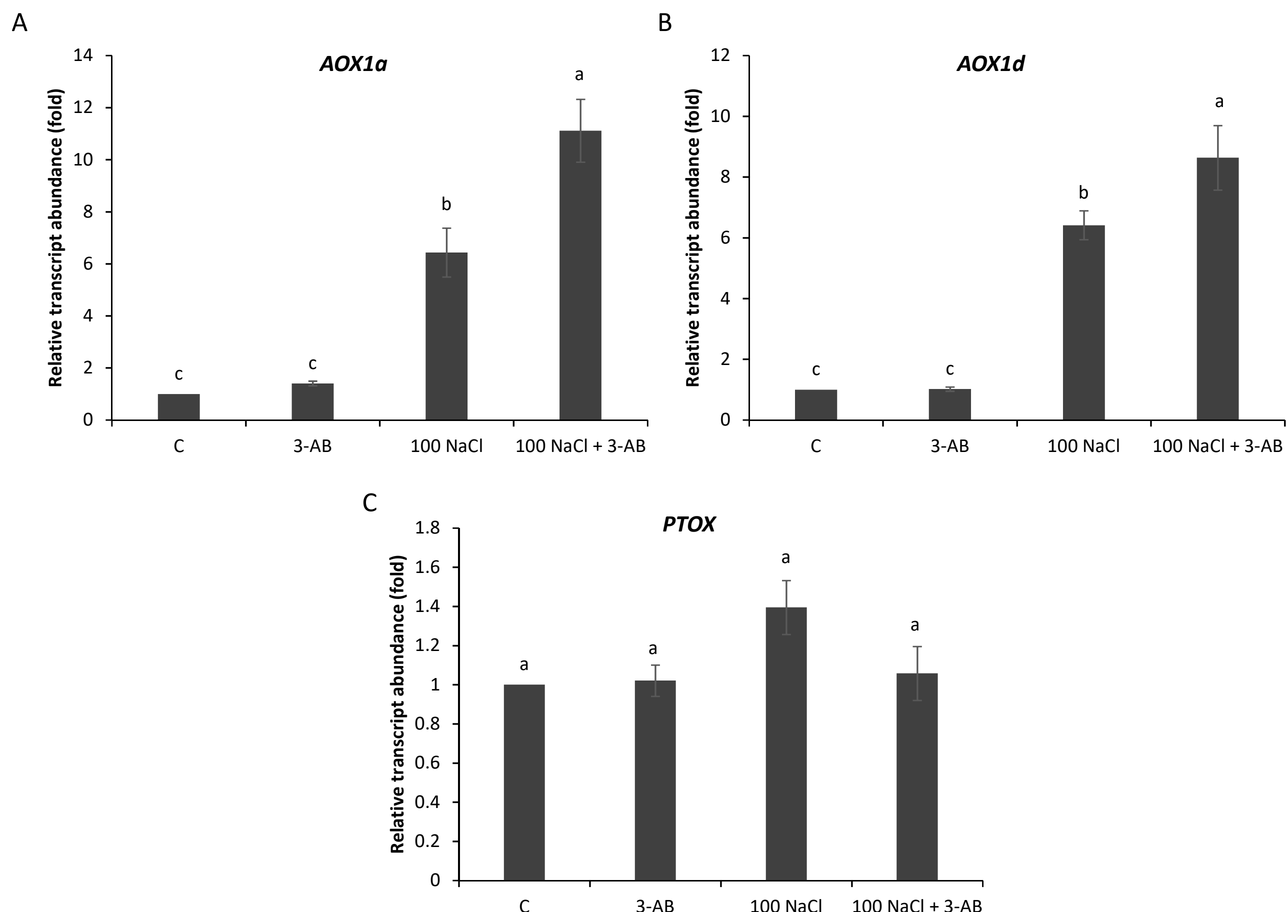

Figure 7. The response of alternative electron pathway related genes to 3-AB under salinity. The expressions of A) *AOX1a,* B) *AOX1d*, and C) *PTOX* genes of *A. thaliana* plants treated with 3-AB and with or without 100 mM NaCl. The Bars = SE.

of five, *AOX1a-d* and *AOX2*) *AOX1a* and *AOX1d* are abundantly expressed and abiotic stress responsive. These results indicate that 3-AB treatment can induce relaxation of mitochondrial electron transfer chain to prevent ROS production. Previously it has been demonstrated that *AOX1a* is involved in stress tolerance to various stresses such as drought, high light (Giraud et al., 2008), low temperature (Watanabe et al., 2008), cadmium stress (Keunen et al., 2016) and arsenic stress (Demircan et al., 2020) by affecting oxidative stress tolerance. Hence induction of *AOX1a* and *AOX1d* by 3-AB under salt stress can be considered as a protective mechanism. However, such phenomenon was not observed for *PTOX*, either in NaCl or NaCl + 3-AB treated plants. Stepien and Johnson (2009) demonstrated that salinity does not induce *PTOX* expression in Arabidopsis unlike other halophyte plants (Uzilday et al., 2015) supporting findings of the current study. However, on top of that, current study demonstrates that 3-AB treatment cannot induce *PTOX* expression similar to induction observed in *AOX* genes.

5. Conclusion

Availability and recycling of NAD^+ are crucial processes to maintain energy homeostasis and both are influenced by PARP inhibition since PARylation acts as a sink for NAD^+. Previously it has been demonstrated that chemical inhibition of PARylation increases NAD^+ content under stressed conditions when compared to nonstress conditions (Schulz et al., 2012). Moreover, plants treated with PARP inhibitors had higher ATP levels and prevented PARylation dependent energy overconsumption with normal levels of mitochondrial respiration leading to lower ROS production (Block et al., 2005). Overall, results of the current study indicate that 3-AB mediated stress tolerance is also related to partial induction of antioxidant defence (SOD) or mitochondrial ROS avoidance mechanisms (upregulated *AOX1a* and *AOX1d*).

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