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Polyamine Oxidase 5 (PAO5) mediated antioxidant response to promote salt tolerance in *Arabidopsis thaliana*

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ABSTRACT

Polyamines are aliphatic polycations ubiquitously found in plants, involved in growth, development and many other physiological processes. Polyamine oxidases are monomeric proteins of about 55 kDa that bear a non-covalently bound molecule of FAD as a cofactor which are responsible for polyamines catabolism. They play very crucial role in polyamine homeostasis. The present study was executed to know the physiological role of polyamine oxidases by using five polyamine oxidase mutants namely *pao1, pao2, pao3, pao4, pao5* along with wild type Col-0. There was almost no difference at physiological condition, but the profound difference was found under salt stress condition. Among the plants *pao5* mutants which is known to have two times higher thermospermine (T-Spm) content exhibited the maximum tolerance under salt stress compared to Col-0 and other mutants. The phenomenon was also confirmed using PAO5 promoter::GUS transgenic plants. At physiological condition, PAO5 promoter activity was observed throughout the plant but intensity is very low, likely, the intensity started to increase gradually once exposed to salt stress and after 9 hours the intensity was very high. Then to reveal the underlying mechanism of stress tolerance, ROS accumulation and antioxidant enzyme activity in *pao5* mutants were measured. *Atpao5* showed almost lower level of ROS accumulation and higher level of antioxidant enzyme activity both in salt stress and control compare to wild type plants. Thus, indicated that lack of *AtPAO5* gene has crucial role in stress tolerance through maintaining the level of T-Spm in *Arabidopsis thaliana*.

Introduction

Polyamines are aliphatic polycations found in all most all living cells. Diamine putrescine (Put), triamine spermidine (Spd) and tetra-amine spermine (Spm) are the common polyamines in plants (Galston and Sawhney, 1990). Some other polyamines such as 1,3-diaminopropane (Dap), cadaverine (Cad), thermospermine (T-Spm), norspermidine (Nor-Spm) and norspermine (Nor-Spm) are also found in many organisms. (Cohen, 1998; Tavladoraki *et al.,* 2011). The different cellular and physiological processes such as growth, organ development, embryogenesis, morphogenesis and leaf senescence are known to be influenced by polyamines in plants (Haque *et al.,* 2018; Walden *et al*., 1997; Malmberg *et al*., 1998; Liu *et al*., 2000; Kusano *et al*., 2007a; Alcázar *et al*., 2006). Cell division one of the important process occurred in presence of high level of polyamine, whereas expansion and elongation of cell occurred in low level of polyamine. They are also involved in vascular development in plants (Galston and Sawhney, 1995).

Polyamine catabolism is very much important for polyamine homeostasis, in which two enzymes, viz. copper-dependent amine oxidase and flavin adenine dinucleotide (FAD)-associated polyamine oxidase (PAO), are involved in catabolism. Maize and barley PAOs are the first characterized apoplastic PAO oxidize polyamines in a terminal catabolic pathway (Ono *et al*., 2011). Recently characterized polyamine oxidases (PAOs) in Arabidopsis and rice showed an alternative pathway of catabolism known as back conversion (BC) pathway. The oxidized product of TC-type PAO, is converted to Nor-Spd and then Nor-Spm by the aminopropyl transferases with broad substrate specificity, which is an established Nor-Spd and Nor-Spm synthesis pathway.

Arabidopsis thaliana genome contains five polyamine oxidase (PAO) encoding genes (*AtPAO1* to *AtPAO5*) for polyamine catabolism (Takahashi *et al*., 2010). Among them *PAO1* and *PAO2* are located in the cytoplasm and others three in peroxisomes (Takahashi *et al*., 2010). All of them are involved in PA back conversion in spite of their different expression pattern and substrate specificity, *AtPAO1* and *AtPAO5* prefer T-spm and back-convert it to Spd, *AtPAO4* is involved in Spm back-conversion to Spd, not to Put, and *AtPAO2* and *AtPAO3* mainly convert Spd to Put (Takahashi *et al*., 2010; Kim *et al*., 2014). A link between PA catabolism and abiotic and biotic stress responses has been

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described Kusano *et al*. (2015), where most of the results were obtained using PAO-specific inhibitors. Growth, development, productivity and geographic distribution of plants become diversified when faced to adverse condition such as drought, salinity, high temperature, nutrient deficiency and others. These stresses cause severe yield loss and reduce almost 50% production of annual and perennial crops worldwide (Wang *et al.,* 2003). Therefore, it is very much important to understand the underlying stress tolerance mechanism in plants to develop innovative approaches to enhance stress tolerance. Synthesis of different metabolites is one of the tolerance mechanisms that plant evolved to overcome the adverse climatic conditions and one good example of stress tolerance is the accumulation of low molecular weight polyamines.

Polyamines and polyamine oxidase genes are involved to different stress response which had already been suggested using model plants *Arabidopsis thaliana*. Polyamine oxidases play crucial roles in stress response such as metal toxicity (Groppa *et al*., 2003), oxidative stress (Rider *et al*., 2007), drought (Yamaguchi *et al*., 2007), salinity (Duan *et al*., 2007) and chilling stress (Cuevas *et al*., 2008). In addition, exogenous application of PAs has also been successfully used to enhance plants tolerance to salinity (Chattopadhayay *et al*., 2002), cold (Nayyar *et al*., 2004), drought (Zeid *et al*., 2004), heavy metals (Wang *et al*., 2007), osmotic stress (Liu *et al*., 2004), high-temperature (Murkowski *et al*., 2001), water logging (Arbona *et al*., 2008) and flooding (Kusano *et al*., 2008). Based on the above information the present investigation was taken to uncover the role of PAOs in salt stress responses in Arabidopsis. In this study, the growth response of different AtPAOs knockout mutants were tested under normal and salt stressed conditions and found that *pao5* mutant was tolerant to salt compare to others. The response of *pao5* to salt stress was also examined using PAO5 promoter:: GUS transgenic plants. To elucidate the underlying molecular mechanism of salt tolerance the antioxidant enzyme activities and reactive oxygen species (ROS) accumulation of *pao5* mutant seedlings in both normal and stressed condition was measured and compared to the wild type one.

Materials and Methods

Plant materials and growth condition

Seeds of *Arabidopsis thaliana* accession Col-0 (WT) and T-DNA insertion lines *Atpao1* (SAIL_822_A11), *Atpao2* (SALK_046281), *Atpao3* (GK209F07), *Atpao4* (SALK_133599), and *Atpao5* (SALK_053110) were used in the study (Sagor *et al*., 2016). Two independent lines of *AtPAO5* promoter::GUS transgenic plant were also used. Seeds of all lines were surface sterilized with 70% ethanol for 1 min and 1% sodium hypochloride with 0.1% Tween-20 for 15 min, followed by extensive washing with sterile distilled water. (Sagor *et al*., 2016). After sterilization, seeds were sown on ½ strength MS which was semi-solidified with plus 1.5% agar plates containing 1% sucrose and B5 vitamin (MP Biomedicals, Cat # 2625149). The plants were grown at 22°C under a 14 h light/10 h dark photocycle at around 60% relative humidity.

Growth response to salt stress

Sterilized seeds of Arabidopsis were grown on ½ MS agar plates containing different concentration of NaCl (0, 25, 50 and 100 mM) and kept in a refrigerator at 4 ^{0}C for 48 hours for seed stratification. Then, the plates were placed at a vertical position with an 85^0 angle and incubated in plant growth room.

Recording of data at different salt concentrated media

Data on germination percentage, root and shoot length, number of cotyledon and true leaf, leaf area and chlorophyll content were recorded as described by Sagor *et al.* (2016).

Histochemical GUS assays

Histochemical GUS assays were performed as described by Jefferson *et al*. (1987) with slight modification according to Sagor *et al*. (2012). Sixteen days old *PAO5* Promoter:: GUS (line 9 and line 10) transgenic plants was detached from MS agar plate and incubated with or without 100mM NaCl solution for 0, 6 and 9 hours. After treatment the plant samples were incubated with GUS staining solution in the dark at 37 ^{0}C for overnight. After incubation, stained plant cells were cleared by 70% ethanol to remove the chlorophyll. Samples were taken on light plate and photographed using digital camera (Canon R250, Japan).

Antioxidant enzyme activity

Activities of catalase (EC: 1.11.1.6), Guaicol peroxidase (EC: 1.11.1.6) and Ascorbate peroxidase (EC: 1.10.3.3) were measured in both normal and 100 mM NaCl treated (12h) Col-0 and *pao5* mutant plants using the standard protocol (Aebi, 1984; Nakano and Asada, 1981).

Determination of H_2O_2 and MDA

Malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) contents were measured according to Heath and Packer (1968).

Statistical analysis

All experiments were performed with at least three biological samples and three time repetition unless mentioned. Data analysis was performed using the Statistical tools (Student's t-test) of Microsoft Excel software.

Results

Sensitivity of polyamine oxidase mutants to salt stress

The growth response of five polyamine oxidase mutant namely *pao1, pao2, pao3, pao4, pao5* and their wild type (Col-0) of *Arabidopsis thaliana* were tested in both physiological and salt stress condition induced by using sodium chloride (NaCl). Both the wild type and mutants lines (*pao1, pao2, pao3, pao4, pao5*) showed varying level of tolerance under salt stress condition. Among the mutants, *Atpao2* showed higher sensitivity whereas *Atpao5* showed the tolerance comparing with the wild type plants (Fig. 1). The level of difference in sensitivity can be explained in terms of germination percentage, root length, shoot length, number of cotyledon and true leaves, chlorophyll content and leaf area. The obtained results are discussed as follows:

Germination percentage

The germination percentage of Col-0, *pao1, pao2, pao3, pao4*, *pao5* were 83.33%, 67%, 16.67%, 75%, 75% and 100%, respectively in normal condition (Table 1). Germination percentage was gradually decreased as the salt concentration increased. *pao1*, *pao3, pao4* mutant showed almost same performance in case of germination percentage at 25 and 50 mM salt concentration (Fig. 1). There is no germination of *pao2* mutant seed as imposed to salt stress regardless the concentration, indicating the highest sensitivity. On the contrary, germination of *pao5* mutant were 100%, 83.33%, 33.33%, 16.67% at 0, 25, 50 and 100 mM salt concentration, respectively (Fig. 1, Table 1)**.**

Table 1. Germination percentage at different salt concentration

Genotypes	NaCl (mM)			
	0	25	50	100
Col-0	83.33	75	33.33	0
pao1	67	75	33.33	0
pao2	14	0	0	0
pao3	75	75	0	0
pao4	75	16.67	16.67	0
pao5	100	83.33	33.33	16.67

Root length and branching pattern

The root length was increased with the increase of salt concentration up to 50 mM (Fig. 2a). The branching pattern of root was also modified. At physiological condition Col-0 and *pao1, pao2, pao3* were slightly branched, whereas *pao4*, *pao5* showed diverse branching pattern. At 25 and 50 mM salt concentration the root length of all the plants were increased comparing with control one, but there was no branching in Col-0, *pao1, pao2, pao3, pao4* plants, and lightly branched in *pao5* mutant plants. The root length at 100mM concentration was maximum in *pao5* mutant followed by *pao4, pao3, pao1*, WT and *pao2* (Fig. 2a).

Shoot length

The shoot length was decreased with the increase of salt concentration (Fig. 2a). In control condition, mutant *pao4, pao5* showed the highest shoot length whereas *pao1* showed almost similar shoot length as wild type plants. Plants of *pao2* mutant showed the poor shoot length among the mutant lines. At 50 mM NaCl containing media *pao1, pao2, pao3, pao4* plants showed poor shoot development, whereas *pao5* plant showed the longest shoot length compare to wild type plants (Fig. 2a). Great difference had been showed among the mutant lines and wild type plants at 100 mM NaCl containing media. There is no shoot development in *pao2* and the well-developed shoot was found in *pao5* mutant (Fig. 2a).

Number of cotyledon leaf

Number of cotyledon leaves was counted from 16 days old plants at 0, 25, 50 and 100mM NaCl containing media. All the plants of both mutants and wild type had two cotyledon leaves at 0, 25 and 50 salt concentration but their sized varied with the increase of salt concentration. At normal condition the cotyledon leaves of both mutants and wild type were large sized, greenish with long petiole (Fig. 2b). But the size became reduced and petiole became short with the increase of salt concentration. At 100 mM salt concentration all plants of *pao5* mutant had two well-developed cotyledon leaves with short petiole but not all the plants of Col-0, *pao1, pao2, pao3*, *pao4* had two leaves (Fig. 2b). Some had no leaf and some had two very small and reduced leaves. Although some plants of wild type and *pao1, pao2, pao3, pao4* mutants had two cotyledon leaves at high salt containing media, those all were very small in size comparing with *pao5* mutants.

Number of true leaf

Number of true leaves were also counted as same as cotyledon leaf. All the plants of both mutants and wild type had large, greenish, long petiolated leaves at normal physiological condition but there was a difference in the number of leaves. At control condition the number of leaves is maximum in *pao5* mutant followed by Col-0, *pao1, pao4, pao2, pao3* (Fig. 2b). In case of 25 mM NaCl containing media also showed similar results, however, in 50 mM NaCl containing media *pao5, pao4*, Col-0 had maximum number of leaves and *pao1* and *pao3* had minimum number of leaves compare to wild type plants. No true leaf was emerged in *pao2* mutant plants at 50 mM salt condition. Only *pao5* mutants showed the development of true leaves in 100 mM NaCl containing media (Fig. 2b).

Chlorophyll content

Chlorophyll content of the true leaves of 16 days old seedlings was measured with the help of SPAD meter.

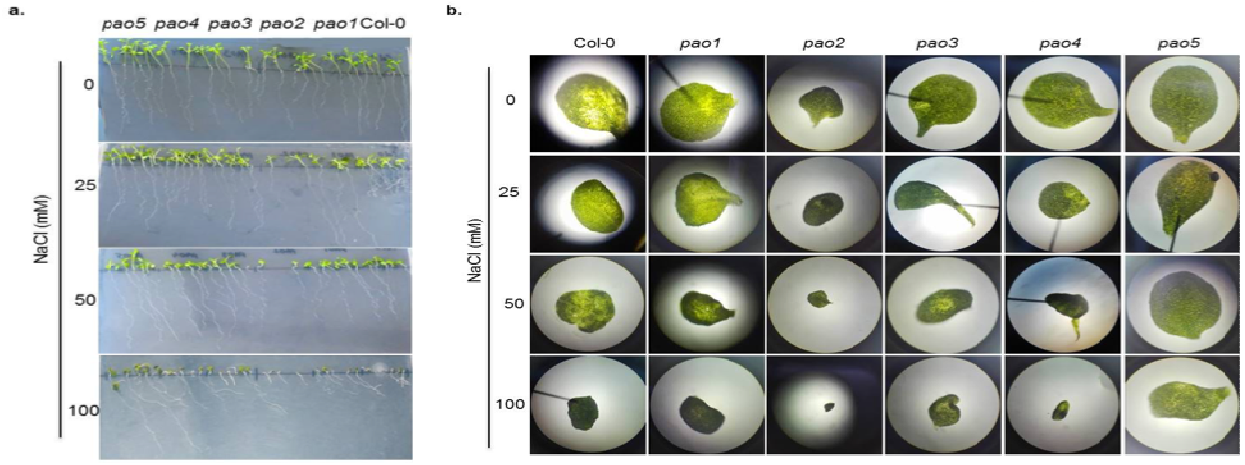

Fig. 1. Growth responses of WT and polyamine oxidase mutants; *pao1, pao2, pao3, pao4* and *pao5* to Salt stress. a) Seeds of WT and *pao* mutants were surface sterilized and sown on 0, 25, 50 and 100 mM NaCl containing MS media, kept the plate in 4^0C for 2 days and then transfer to normal growth condition. The pictures were taken two weeks after germination. b) Leaf of WT and mutants were detached and put under digital microscope to take individual pictures.

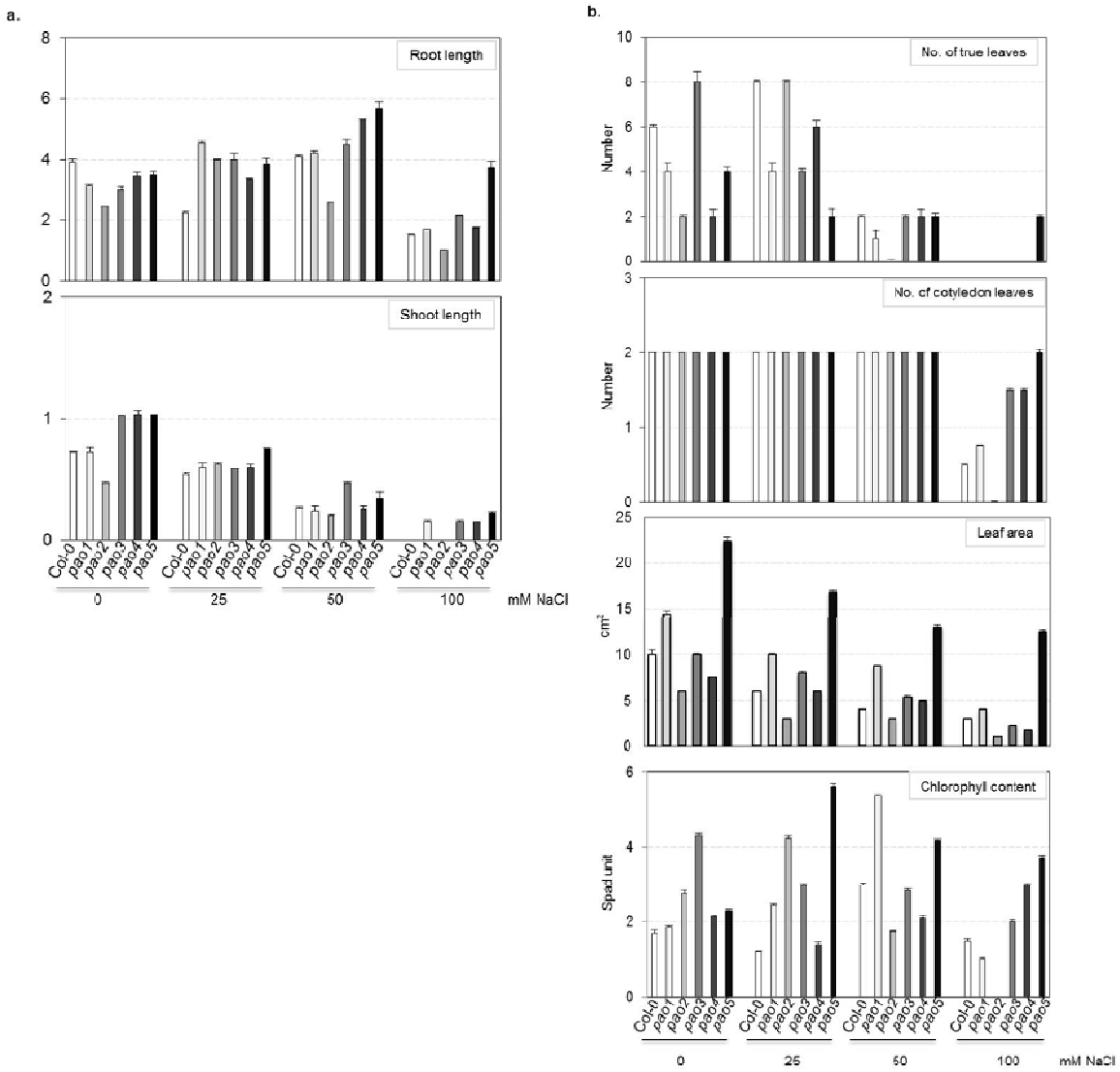

Fig. 2. Different growth parameters of WT and *pao* mutants grown on MS agar medium containing 0, 25, 50, and 100mM NaCl. a) root and shoot length b) number of true and cotyledon leaves, leaf area and chlorophyll content of true leaves. The values indicate means ± SE (Standard Error).

The highest value was observed in pao3 at physiological condition followed by pao2, pao5, pao4, pao1 and Col-0 (Fig. 2b). In line with the exposure to salt stress, different mutant showed different value. Chlorophyll content of pao5 mutant was increased with the increase of salt concentration whereas pao3 went for in opposite direction (Fig. 2b). In case of pao1, pao2, pao4 mutant plant, the chlorophyll content value varied at different level at different salt concentration without any significant indication.

Leaf area

Leaves of both wild type and mutant plants were large, greenish, straight without any curl and long petioled under non-stressed condition. But leaf area started to decrease gradually as the salt concentration started to increase. The maximum leaf area was found in *pao5* mutant whereas in *pao2* was minimum under both stressed and non-stressed condition comparing with wild type plants (Fig. 2b). The rate of decrease of leaf area was minimum in *pao5* mutant plants followed by Col-0, *pao1, pao3, pao4, pao2 (*Fig. 1b).

AtPAO5 promoter:GUS expression analysis

To confirm the involvement of polyamine oxidase-5 in salt stress response, promoter::GUS transgenic approach was used. Two week old seedlings of the *PAO5* promoter::GUS transgenics were exposed to high salt stress for 0, 6, 9 h and assayed for GUS staining. The intensity of GUS staining was weakened (Fig. 3) at physiological condition, and gradually increased with the time after exposure to salt stress. At physiological condition, GUS gene expression was found in leaf, cotyledon and root also (Fig. 3), but the intensity is very low. When the plant is exposed to salt stress, the intensity of GUS staining was increased gradually and strongly induced after 9 h in all the examined parts including leaf, cotyledon and root also. Among the different parts, the expression is stronger in true and cotyledon leaves indicating the tolerance response to salt stress.

H_2O_2 and MDA contents

Salt stress generally leads to an overproduction of ROS like H_2O_2, MDA which are responsible for oxidative stress. Exposure of wild type Col-0 and *pao5* mutant plants to salt stress resulted in increase in the accumulation of H_2O_2 up to 9 hours treatment under both stressed and non-stressed condition. After 9 hour, under non-stressed condition the activity of H_2O_2 decreased but the level was almost same for both wilt type Col-0 and *pao5*. In case of stressed condition, H_2O_2 activity was higher in wild type Col-0 but lower in *pao5* after 9 hours treatment (Fig. 4a). In both stressed and non-stressed condition, the activity of H_2O_2 was lower in *pao5* mutants than the wild type Col-0 (Fig. 4a) suggesting that *pao5* may have some protective mechanism to overcome salt-induced oxidative stress by maintaining lower level of H_2O_2. The MDA activity fluctuated with time in wild type Col-0 plants but notably decreased in *pao5* mutants under non-stressed condition (Fig. 4a). In case of stressed condition MDA activity found much higher in wild type compare to *pao5* mutant (Fig. 4a) indicating that *pao5* mutants can withstand against stress condition.

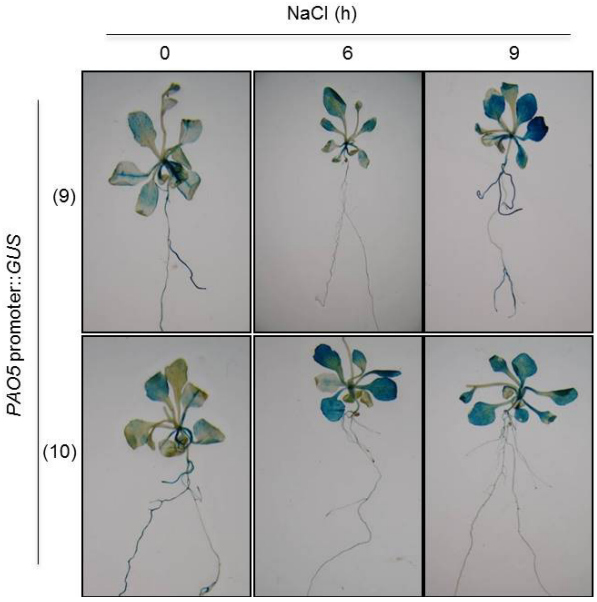

Fig. 3. Salt induced *PAO5* expression analyzed by GUS staining using *PAO5* promoter::GUS transgenic plants. Two week old promoter GUS transgenic plants were detached from MS agar plate, incubate wet filter paper containing 200 mM NaCl solution for 0, 6 and 9 hours and then transfer to GUS staining solution and incubate at 37 ^{0}C overnight. Pictures were taken using sony digital camera

Antioxidant enzyme activity

In the current study APX, POD and CAT activities was measured for both salt-stressed and non-stressed *Arabidopsis* plants. At stress-free condition, the APX activity in *pao5* mutant is consistently higher compare to wild type Col-0 plants (Fig. 4b) regardless the time of treatment. After exposure to salt stress, the APX activity slightly increased in *pao5* mutant plants compared to non-stressed plants, but still found higher than wild type regardless time and treatment (Fig. 4b). The POD activity in *pao5* mutant was much higher compare to wild type Col-0 both in both non-stress and salt-stress condition (Fig. 4b). The CAT is one of the most effective antioxidant enzymes in preventing oxidative damage. In our study, the CAT activity in wild type plant is comparatively lower than that of *pao5* mutant at physiological condition up to 9-hour treatment (Fig. 4b). Under salt-stressed condition, *pao5* showed much higher CAT activity than wild type Col-0 plants with time.

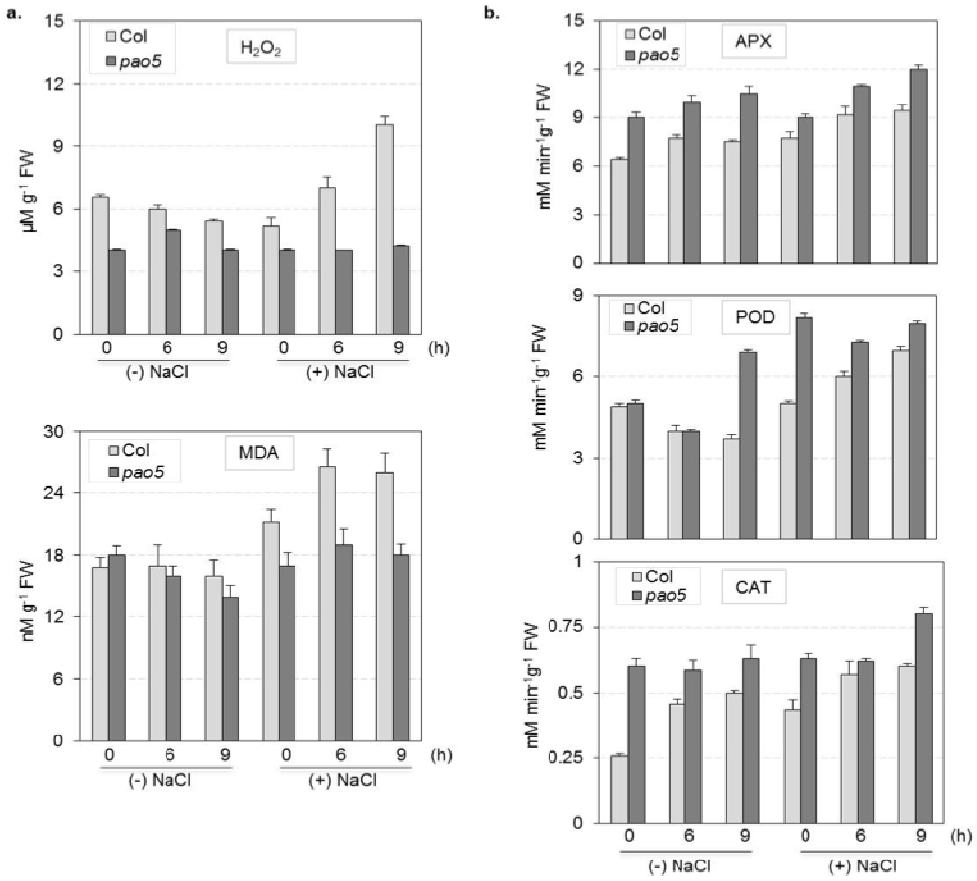

Fig. 4. ROS accumulation and antioxidant activity in response to salt stress. a) MDA and H_2O_2 content b) APX, POD and CAT activity in wild type Col-0 and *pao5* mutant under both stressed and non-stressed condition. Two week old seedlings of wild type and *pao5* mutant were carefully detached from ½ strength MS media and incubate in wet filter paper with or without 200 mM NaCl for 0, 6 and 9h under normal light condition. Plant samples were collected and used for measuring antioxidant activity. Error bar indicates mean ± SE.

Discussion

Polyamines are positively charged molecules ubiquitously found in plants involved in different physiological processes. Polyamine homeostasis in cell is very much important which become maintained through biosynthesis and degradation. Biosynthetic pathway of polyamines are well established in contrast to degradation one which is poorly understood. Degradation reaction of polyamines in cell is catalyzed by major enzyme polyamine oxidases (PAOs). The present study was planned to reveal the protective role of polyamine oxidases in salinity tolerance condition using wild type and different polyamine oxidase mutants namely *pao1, pao2, pao3, pao4, pao5* of *Arabidopsis thaliana.*

First the phenotypic performances of both wild type and mutant plants were evaluated under physiological and stressed condition. There is no significant difference between wild type and mutant plants at physiological condition, whereas, salt stress condition showed significant phenotypic variation. Both the mild (25 and 50 mM) and strong stress (100 mM NaCl) , data on different parameters like germination percentage, root length, shoot length, number of cotyledon leaf and true leaves, leaf area and chlorophyll content were investigated. The single *pao5* showed the highest germination percentage whereas *pao2* showed the lowest under both physiological and stressed condition. Shoot length and root length were also higher in *pao5* plants comparing with wild type in both conditions. Besides, the number and size of true leaves and cotyledon leaves were also maximum in *pao5* mutants and minimum in *pao2* in respect of in almost all parameter. The rest of the mutant *pao1, pao3, pao4* showed average performance comparing with control plants. Leaf area was also the greater in *pao5* plants than wild type and other mutants under both stressed and non-stressed condition. Thus, among the plants, *pao5* mutants exhibit the most tolerance compared with other mutants and wild type plants under salt stressed condition. As *pao5* mutants are produced by knocking down the *AtPAO5* gene which is responsible for T-spm catabolism, T-spm remains two or three-fold higher in this mutants (Ahou *et al*., 2014; Kim *et al*., 2014; Tavladoraki *et al*., 2016), thus it may have some underlying mechanisms to withstand under stressed condition. Zarza *et al.* (2017) showed the accumulation of tSpm in pao5 mutant trigger metabolic and transcriptional reprogramming to promote salt stress tolerance. It may suggest that *pao2* mutants which was produced by knocking down the *AtPAO2* gene responsible for to catalyze spm and spd, but not T-

spm, have less defense mechanism under stressed condition due to loss of some intracellular components due to no change in the T-spm content. To reveal the role the polyamine oxidases (PAOs) in stress response an attempt was made by Sagor *et al*. (2016) and suggested that cytoplasmic PAOs (*pao1/pao5*) silenced mutants showed salinity tolerance by reducing ROS production and up-regulating the expression of a subset of stress responsive genes under salt stress condition. Kakehi *et al*. (2010) showed that endogenous T-Spm level is very much important for normal growth of plants and the altered phenotype of T-Spm deficient mutant can be functionally substitute by another tetraamines. Exogenous thermospermine at higher concentration compare to its basal level induce a number HR related defense genes and also protect the CMV coat protein accumulation in Arabidopsis leaves (Sagor *et al*., 2012). The polyamine oxidase 5 genes is expressed in different parts such as xylem, phloem, cambial cells, pericycle (Fincato *et al*., 2012; Ahou *et al*., 2014), hypocotyls (Fincato *et al*., 2012). T-Spm is not a minor polyamine in plants and they are highly linked with the expression of genes realted to auxin and cytokinin signaling pathways (Takahashi *et al.,* 2012; Ren *et al*., 2009; Perilli *et al*., 2010; Bishopp *et al*., 2011). That evidence strongly supports the importance of T-Spm at molecular and cellular level.

To further confirm the involvement of polyamine oxidase-5 in salt stress response, promoter::GUS transgenic approach was used. The intensity of GUS staining was weakened at physiological condition, and gradually increased with the time after exposure to salt stress. At physiological condition, GUS gene expression was found in leaf, cotyledon and root also, but the intensity is very low. Once the plant is exposed to salt stress, the intensity of GUS staining was increased gradually and strongly induced after nine hours in all the plant parts including leaf, cotyledon and root also. Among the different parts, the expression is stronger in true and cotyledon leaves indicating the tolerance response to salt stress. These findings is consistent with the previous findings, where the promoter:: GUS activity of spermine synthase gene also found to be intense in cotyledon leaves upon 12 h of salt treatment (Sagor *et al.,* 2011).

To reveal the underlying biochemical mechanism of salt tolerance in *pao5* mutants, we studied the antioxidant response in terms of ascorbate peroxidase (APX), catalase (CAT), guaicol peroxidase (POD) activity and also accumulation of hydrogen peroxide (H_2O_2) and melondealdehyde (MDA) as they are known to play critical role in salt response (Radi *et al*., 2013; Esfandiari and Gohari, 2017). We documented lower accumulation of ROS (H_2O_2 and MDA) under salt-stressed condition in *pao5* mutants than wild-type Col-0 plants. It may suggest that, *pao5* may play critical role to withstand under stressed condition by producing no significant higher level of ROS contents compared with wild-type plants. Reactive oxygen species (ROS) overproduction due to salt stress can target DNA mutation, degradation of protein, membrane instability, ion channel activity in plant cells (Temizgul *et al*., 2016). Salt stress can also make an imbalance in antioxidant activity (Kaya *et al*., 2015) and make an increase of H2O2 and MDA in sensitive species (Radi *et al*., 2013; Esfandiari and Gohari, 2017). Reactive oxygen species (ROS) plays a dual role in cells, they can acts either as signaling molecule in response to stress condition at lower level, but once overproduced become toxic to cell cause death of the cell (Mittler, 2002; Miller *et al*., 2010). Polyamine oxidase gene (PAOs) from Arabidopsis thaliana and Oryza sativa are involved in the back conversion of polyamines yielding the production of H_2O_2 both in vitro and in vivo (Tavladoraki *et al*., 2006; Kamada-Nobusada *et al*., 2008; Moschou *et al*., 2008; Ono *et al*., 2011; Ahou *et al*., 2014; Liu *et al*., 2014) and also act as signaling component in response to tolerance against tobacco mosaic virus (TMV) infection (Takahashi *et al*., 2003). These results also support the current analysis indicating that *pao5* mutant can maintain lower level of H_2O_2 and MDA through maintaining higher level of T-spm.

Over-accumulation of ROS cause oxidative damage in cells which are normally coped through the up-regulation of antioxidant enzyme (APX, CAT, POD) activity in plants (Wang *et al*., 2009). Here in the present study, at both normal and salt-stressed condition, the APX activity in *pao5* mutant is consistently higher compare to wild-type Col-0 plants regardless the time of treatment (Fig. 4b). The POD activity in pao5 mutant is much higher compare to wild-type Col-0 both in physiological and salt stress condition (Fig. 4b). Also, CAT activity in wild type plant is comparatively lower than that of pao5 mutant under salt-stressed condition (Fig. 4b). APX and CAT both are involved in the degradation of H_2O_2 to H_2O to reduce oxidative damage in cells (Fridovich, 1989; Caverzan *et al*., 2012; Hossain *et al*., 2013). Changes in the activity of antioxidant enzymes upon salinity has been reported in different plants such as CAT in soybean (Comba *et al*., 1998) tomato (Rodriguez-Rosales *et al.,* 1999; Al-aghabary *et al*., 2005), and mulberry (Sudhakar *et al.,* 2001); APX and CAT in alfalfa (Wang *et al*., 2009); CAT, POD and APX in Plantago (Radyukina *et al*., 2009). Thus the higher or consistent level of antioxidant enzyme activity in *pao5* mutants may play a crucial role in salt stress tolerance through maintaining the level of T-Spm in *Arabidopsis thaliana*.

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