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Assessment of Salt Stress Effects on Antioxidant Levels and Membrane Transport Protein in *Amaranthus caudatus*

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Abstract

Salt stress is a major environmental challenge for plants, leading to various physiological and biochemical responses. This study investigates the impact of salt stress on lipid peroxidation, phenolic and flavonoid accumulation, free-radical scavenging antioxidant activity, glutathione content, tocopherol levels in three amaranth (*Amaranthus caudatus* L) genotypes: Red Cascade (Red), Green Cascade (Green), and Pony Tail Mixed (Pony). The results showed that, under salt stress, lipid peroxidation increased, causing cell membrane damage and compromising cell integrity. However, salt stress also triggered the accumulation of phenolic and flavonoid compounds, which possess strong antioxidant properties and play a crucial role in scavenging reactive oxygen species. The total antioxidant activity as measured by DPPH inhibition was significantly enhanced in all genotypes under salt stress, with the Green genotype showing the highest activity. Additionally, salt stress induced an increase in total glutathione levels, maintaining the GSH/GSSG ratio relatively constant in all genotypes. However, tocopherol content decreased significantly under salt stress, with the Green genotype being the most affected. Finally, as membrane compounds are affected by oxidative stress, an analysis of membrane trafficking in root epidermal cells revealed that the Pony genotype had the highest response of the three genotypes. Overall, these findings suggest that salt stress induces complex responses in *A. caudatus* genotypes, involving oxidative damage, phenolic and flavonoid accumulation, enhanced antioxidant activity, and alterations in tocopherols and enhanced membrane trafficking. Understanding these responses can contribute to the development of salt-tolerant crops and improve agricultural productivity under saline conditions.

Keywords Abiotic stress · Amaranth · Oxidative stress · Metabolic mechanisms · Membrane trafficking

Introduction

Over the past few decades, it has become apparent that climate change is one of the most serious problems for agriculture and food security. Abiotic stress factors, including fluctuations in temperature, high-light, floods, salinity, droughts, hazardous and toxic chemical-polluted soils, heavy metals, etc., have an increasing impact on plant development as a result of global warming (Hussain et al. 2019; Singh et al. 2019). In addition, variations in temperature and precipitation have a significant effect on soil salinity. In fact, the thermal expansion of sea water and the melting of ice sheets increase sea levels, which causes floods and salinity of surface and groundwater as well. It has been reported that in dry landscapes, rising temperatures and falling precipitation are correlated with rising soil salinity (Bannari and Al-Ali 2020). For instance, salinity in coastal agricultural fields has increased from 1 to 33% over the 25 last years due to

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the increased sea level (Corwin 2021). Therefore, salinity is one of the most frequent and complex abiotic stressors that threaten agriculture worldwide. In fact, high salinity affects around 50% of irrigated and cultivated agriculture land (Gengmao et al. 2015). Most of the subsurface water used for crops turns brackish because of the high concentration of soluble salt ions which causes oxidative stress (Caverzan et al. 2016). Moreover, stomatal conductance decreases in response to salt stress, which affects the redox balance in the organelles of the stressed plants (Corpas et al. 2015). For example, the hyper ionic salt stress in the root decreases the uptake of several minerals such as potassium, calcium, and magnesium, leading to disturbance in stomatal conductance. As a result, the production of reactive oxygen species (ROS) declines the availability of carbon dioxide in the stressed seedlings and increases the photorespiration rates (Kangasjärvi et al. 2012). In addition, ROS cause various physiological damages in plants, such as lipid peroxidation, disruption of the photosynthetic apparatus and pigments, Calvin-cycle dysfunction, electron-transport-chain (ETC) efficiency reduction, $NADP^+$ regeneration and even mutagenesis through DNA damages, which adversely impact the crop growth and yield (Hasanuzzaman et al. 2017).

In response to salt stress plants develop several defense mechanisms by ROS-scavenging enzymes and antioxidants (Singh et al. 2019). In fact, to mitigate the excessive ROS production, plants accumulate various enzymatic and non-enzymatic antioxidants. Antioxidant enzymes include peroxidase, superoxide dismutase, glutathione reductase, ascorbate peroxidase, etc. In this sense, (Mittova et al. 2004) reported that 100 mM NaCl treatment induced antioxidant enzymes activities, such as superoxide dismutase and ascorbate peroxidase in *Solanum pennellii* seedlings, which decreased lipid peroxidation. Nevertheless, the non-enzymatic antioxidants include the plant secondary metabolites, such as red pigments, carotenoids, polyphenols (phenolic acids and flavonoids), and glutathione (GSH) (Sarker and Oba 2018). For instance, previous studies have shown that flavonoid biosynthesis is induced by salt stress, which enhances salt stress tolerance through the scavenging of free radicals (Chandran et al. 2019).

Amaranths are C4 plants with mechanisms that enable them to grow in water- and nutrient-limited environments. They exhibit strong capacity to grow under unfavorable conditions, especially in saline environments(Huerta-Ocampo et al. 2014; Pulvento et al. 2015; Tebini et al. 2022a). Due to its high nutritional value and ability to withstand stress, amaranths have emerged as an alternative for sustainable food production facing climate change. Soil salinization constitutes a global challenge, leading to yield losses in most crop plants (Chourasia et al. 2021). Amaranth has a high nutritional value and contains wide range of essential nutrients, including vitamins A, B (thiamine, riboflavin, niacin and folate) and C, as well as minerals such as calcium, magnesium, potassium, phosphorus, iron, zinc, copper and manganese (Chakrabarty et al. 2018). In addition, amaranth leaves contain various bioactive compounds such as pigments, phenolic compounds and flavonoids (Sarker et al. 2018).

This study aimed to understand the mechanisms of salt stress tolerance by investigating metabolic mechanisms, including levels of secondary metabolites in mature plants of three A. caudatus genotypes. In addition, since the oxidative stress generated by salt stress has an impact on membrane components, membrane behavior, such as trafficking, was investigated.

Materials and Methods

Plant Materials and Growth Condition

Three genotypes of *Amaranthus caudatus* L., 'Red Cascade' (Red), 'Green Cascade' (Green) and 'Pony Tail Mixed' (Pony) distinguished by their tolerance to salinity, previously described by (Tebini et al. 2022a), were used. Seeds were sterilized with a solution of calcium hypochlorite (20%). After 2h imbibition, seeds of each genotype were germinated in Petri dishes on two layers of water-saturated filter paper at 25 ± 2 °C for 7 days. The germinated seedlings with two cotyledons were transferred to hydroponic conditions in black plastic trays filled with 2.5 L of Hoagland nutrient solution (3 mM KNO_3, 1 mM $Ca(NO_3)_2$, 2 mM KH_2PO_4, 0.5 mM $MgSO_4$, 32.9 µM Fe-Ethylenediamine-tetraacetic acid (EDTA), 30 µM H_3BO_4, 5 µM $MnSO_4$, 1 µM $CuSO_4$, 1 µM $ZnSO_4$, and 1 µM $(NH_4)_6Mo_7O$) (Hoagland and Arnon 1950). Plants were grown with nutrient solutions for 20 days in an air-conditioned chamber under an air humidity ranging from 60 to 80% with artificial light (150 µmol m^{-2} s^{-1}; 16 h photoperiod) at 25 ± 2 °C during the day and 20 ± 2 °C at night.

Salinity Stress Treatment

Salt treatments have been applied on 30-day-old seedlings at the stage of three leaves. Three treatments were applied.

(i) 0 mM NaCl: Nutrient solution without NaCl,
(ii) 100 mM NaCl: Nutrient solution supplemented with either 100 mM NaCl,
(iii) 200 mM NaCl: Nutrient solution supplemented with either 200 mM NaCl.

In addition, the pH of the nutrient solution was adjusted at 6 with KOH (1N). Salt was added progressively at a rate of 50 mM per day to avoid osmotic shock to the plant. After 15

days under salt treatments plants were harvested and leaves were separated and immersed in liquid N_2 to preserved at − 80 °C until analysis. Three plants per treatment and per genotype were analyzed.

Determination of Lipid Peroxidation

The level of lipid peroxidation (mainly malondialdehyde (MDA)) was measured according to (Madhava Rao and Sresty 2000). Briefly, 250 mg of fresh leaf were homogenized with 5 mL of trichloroacetic acid 5% (w/v) and centrifuged at 12,000 g for 15 min at 4 °C. 2 mL of the supernatant was mixed with 2 mL of 0.67% (w/v) thiobarbituric acid and the mixture was incubated for 30 min at 100 °C and then quickly cooled with ice. After centrifugation (10,000 g for 1 min at 4 °C), the optical absorbance of the mixture was read at 532 and 600 nm. The MDA concentration was calculated using its molar extinction coefficient (155 mM^{-1} cm^{-1}).

Volatile Organic Compounds Extraction

The extraction of volatile organic compounds was carried out by the maceration technique (Tebini et al. 2022b). Briefly, 500 mg of leaf powder of three amaranth genotypes were weighed and homogenized with 2 ml of methanol 80% (1:2 w/v) using a chilled blender for 5 min. The sample was homogenized again using a Polytron homogenizer for a further 3 min. The homogenates were filtered through Whatman No. 2 paper on a Buchner funnel under vacuum. Then the methanolic extract was collected and frozen at − 20 °C until analysis.

Total Phenolic Content

The total phenolic content (TPC) was assessed according to Singleton et al. (1999). Briefly, an aliquot of 250 µl of methanolic extract was added to 3500 µL of distilled water and 250 µL of Folin-Ciocalteu reagent. After incubation for 3 min at room temperature, 1 mL Na_2CO_3 was added, and the tube was vigorously shaken and incubated for 40 min at 40 °C in a water bath and the absorbance was measured at a wavelength of 760 nm. The obtained data were expressed in mg of gallic acid equivalents per gram of fresh weight of each sample (GAE/g^{-1} FW).

Total Flavonoids Content

The total flavonoids content (TFC) of methanolic extracts were determined by method of Dewanto et al. (2002) with slight modifications. Briefly, 1000 µL of the methanolic extract was added to 1000 µL of $AlCl_3$ (2%). After incubation for 10 min in a dark room, the optical absorbance was measured at 440 nm. The obtained data were expressed in mg of quercetine equivalents per gram of fresh weight of each sample (QE/g^{-1} FW).

Radical Scavenging Activity

The radical scavenging activity in three amaranth genotypes leaves were determined through free radical scavenging activity, i.e. the ability to scavenge 2,2-diphenyl-picryl-hydrazyl (DPPH) free radicals, and expressed by the percentage of inhibition of methanolic extracts (IP%), as described by Sharma and Bhat (2009). Briefly, 20 µL of methanolic extract were added to 1 mL of 50 µM DPPH dissolved in methanol. The mixtures were incubated in the dark for a period of 20 min. Ascorbic acid was used as positive control and the DPPH absorption was determined at 515 nm.

Total Antioxidant Activity

The total antioxidant activity (AAT) is based on the reduction of molybdenum VI to molybdenum V by the substrate. The reduction leads to a decrease in pH and the formation of green colored phosphate-molybdenum V complex (Prieto et al. 1999). Briefly, 0.1 mL of methanolic extract was mixed with 1 mL of a solution composed of sulphuric acid (H_2SO_4; 0.6 N), sodium phosphate (NaH_2PO_4, H_2O; 28 mM) and ammonium molybdate ($(NH_4)_6Mo_7O_{24}$, $4H_2O$; 4 mM). The samples were incubated at 95 °C for 90 min. After 6 min of incubation at room temperature, the absorbance was measured at 695 nm. The total antioxidant capacity is expressed as mg gallic acid equivalent per gram of fresh weight (mg GAE/g FW).

HPLC Method for the Analysis of α-Tocopherol

The concentration of α-tocopherol and its oxidation product α-tocopherol quinone were estimated as detailed by Munné-Bosch et al. (2007) after extraction in ice-cold n-hexane containing 1 ppm butylated hydroxy toluene and separation on a Partisil 10 ODS-3 column at a flow rate of 1 mL min^{-1} with a methanol/water (95:5, v/v) solvent. The α-tocopherol and α-tocopherol-quinone were detected at 283 and 265 nm, respectively.

HPLC Glutathion Assay

Reduced glutathione (GSH) and total glutathione (GSH + GSSG) were quantified after HPLC and *ortho*-phthalaldehyde (OPA) pre-column derivatization according to Cereser et al. (2001): OPA derivatives were separated on a reversed-phase HPLC column (C18 Pyramid Column) with an acetonitrile–sodium acetate gradient system at pH 6.2 and 30 °C with a flow of 0.7 mL min^{-1}. Fluorimetric detection

was performed with a fluorescence Shimadzu RF-0A detector at 420 nm after excitation at 340 nm.

Microscopic Observation

In order to investigate the early events of membrane dynamics upon salt stress, we used cell staining with the styryl dye FM4-64 (Luu et al. 2012). Seeds of three amaranth genotypes were germinated in 14 cm diameter Petri dishes lined with a double layer of filter paper. After seven days of germination, seedlings with two-cotyledons age were separated into two conditions: a control first group including the non-treated seedlings and a second group with seedlings treated with 200 mM NaCl for 10 min. The seedling roots in each group were also stained with FM4-64 for five minutes. For each condition, 20 to 46 cells were analyzed from at least three different seedlings. Microscopic observation was performed with an Apotome 2 or Epifluorescence microscope equipped with a structured illumination system to improve axial resolution in fluorescence mode Z stack acquisition, mosaic, timelapse, Source: Xcite 120 LED with a BP 538/40 BS FT 570 em BP 600/50 filter (http://www.zeiss.com/). A 63X (1.4 NA) lens was used with a digital zoom setting. Images were collected at 128 pixels with a scan speed of 0.246 s/image. After being taken, the images (in Z-stak) were deconvoluted by Huygens software. Fluorescence signal was quantified using Image J software (Rasband W.S., NIH, http://imagej.nih.gov/ij/) which allows the measurement of the average grey value in each cell. Using excels, the results of the FM4-64 labelled cells and the mean grey value were presented in graphical form.

Statistical Analysis

A two-way analysis of variance (ANOVA) with amaranth genotype and salt treatment as factors, and their interaction was performed for the whole dataset using the XLSTAT software. The means were compared using Duncan's test at $p < 0.05$ when significant differences were found. The correlation analysis was performed using the correlation test and linear regression program in the XLSTAT program.

Results

Salt Stress Increases Lipid Peroxidation and Cell Membrane Damage

A significant increase in MDA levels in the leaves of three amaranth genotypes was observed with increasing NaCl (Fig. 1) (Table 1). Moreover, the increase in MDA levels was similar for the three different genotypes. In fact, for all genotypes, MDA accumulation increased from around 0.6–0 µmol g^{-1} FW to 0.9 µmol g^{-1} FW in response to 100 mM NaCl treatment. Interestingly, in response to 200 mM NaCl treatment, MDA concentration increased to 2.1 µmol g^{-1} FW, which is threefold higher than the control. The data indicate that MDA accumulated and increased similarly in all three lines in response to salt stress. Thus, salt stress induced membrane lipid peroxidation, which can lead to oxidative damage in membranes.

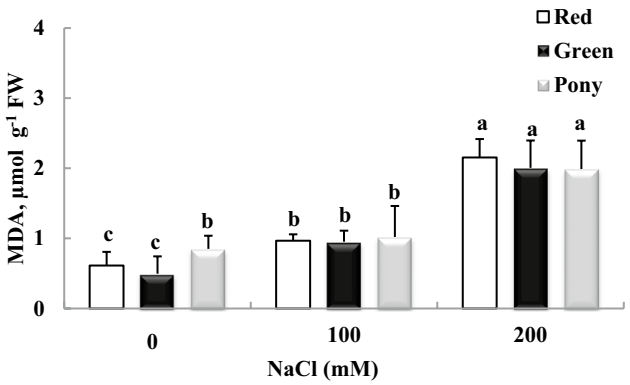

Fig. 1 Malondialdehyde content (MDA) in three amaranth (*Amaranthus caudatus* L.) genotypes subjected to salt treatments (0, 100 and 200 mM NaCl). Data are means of 3 replicates ± SE and values followed by at least one letter are not statistically different at $p < 0.05$ according to Duncan's test

Salt Stress Enhanced Phenolic and Flavonoid Accumulation

Next, we monitored the impact of different salinity concentrations on TPC. Under control condition (0 mM NaCl), the three genotypes showed similar values of TPC (~ 90 mg GAE g^{-1} FW). However, after salt- treatment, TPC was progressively enhanced in leaves. In fact, the analysis of the methanolic extracts revealed that the Red and Pony genotypes had high levels of TPC (~ 152 mg GAE g^{-1} FW) after treatment with 200 mM NaCl. The increases were in the order of 70% and 63% compared to the control, respectively (Fig. 2a). In contrast, Green genotype exhibited the lowest total polyphenol content (110 mg GAE g^{-1} FW) among the genotypes.

Similarly, in control condition the three genotypes showed similar values of TFC (~ 7 mg GAE g^{-1} FW) (Fig. 2b) (Table 1). However, under salt treatment, TFC was progressively enhanced in leaves. Indeed, a 2.2- and 2.5-fold increase of TFC was observed in Red and Pony genotypes at 100 mM compared to the control, respectively. Interestingly, the data showed that TFC were higher in Red and Pony genotypes than in Green genotype following the increasing salinity treatment. Indeed, after 100 mM NaCl treatment, the highest TFC was detected in Pony genotype (21 mg GAE g^{-1}

Table 1 Summary of analysis of variance for 14 biochemical traits of three amaranth (*Amaranthus caudatus* L.) genotypes subjected to salt treatments (0, 100 and 200 mM NaCl)

Parameters	Source of variation					
	Genotype		Treatment		Genotype × Treatment	
	F-value	Pr > F	F-value	Pr > F	F-value	Pr > F
MDA	3.175	0.066	355.646	< 0.0001	3.083	0.043
GSH_T	22.241	< 0.0001	36.488	< 0.0001	13.117	< 0.0001
GSH_R	8.277	0.003	93.489	< 0.0001	47.841	< 0.0001
GSSG	16.069	< 0.0001	63.699	< 0.0001	7.869	0.001
GSSG/GSHratio	3.132	0.068	7.329	0.005	1.031	0.418
Polyphenols	26.867	< 0.0001	145.220	< 0.0001	7.595	0.001
Flavonoids	82.732	< 0.0001	315.910	< 0.0001	17.548	< 0.0001
DPPH test	122.370	< 0.0001	21.486	< 0.0001	290.407	< 0.0001
AAT	49.410	< 0.0001	135.513	< 0.0001	4.598	0.010
α	1.514	0.247	53.3415	< 0.0001	5.600	0.004
β	104.694	< 0.0001	9.728	0.001	4.830	0.008
γ	0.131	0.878	59.459	< 0.0001	33.859	< 0.0001
δ	18.142	< 0.0001	25.375	< 0.0001	54.351	< 0.0001
Tocopherol	4.281	0.030	64.757	< 0.0001	15.886	< 0.0001

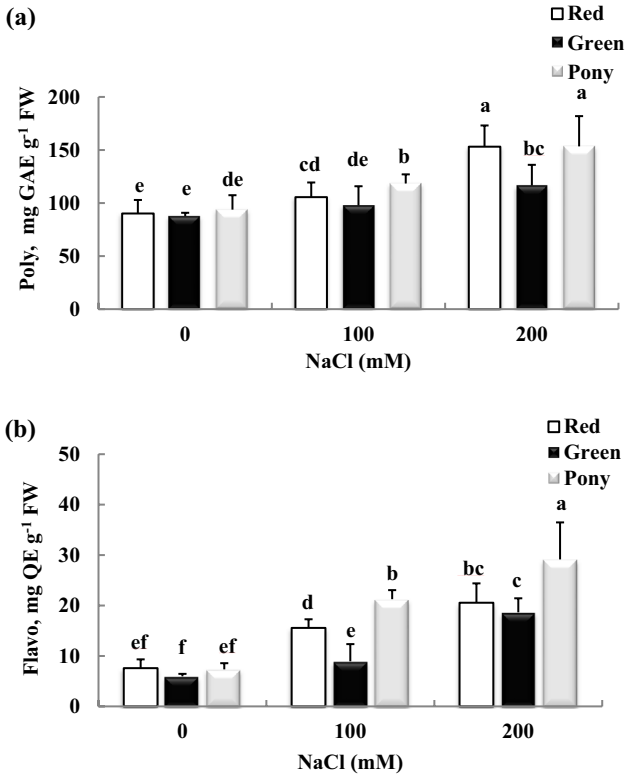

Fig. 2 Total polyphenol content (TPC) (**a**) and total flavonoid content (TFC) (**b**) in three amaranth (*Amaranthus caudatus* L.) genotypes subjected to salt treatments (0, 100 and 200 mM NaCl). Data are means of 3 replicates ± SE and values followed by at least one letter are not statistically different at $p < 0.05$ according to Duncan's test

FW) followed by the Red genotype (16 mg GAE g^{-1} FW). In contrast, the lowest value was found in the Green genotype (8 mg GAE g^{-1} FW). In addition, under 200 mM NaCl the TFC increased significantly in Green, Red and Pony genotypes compared to the control: an approximately sixfold increase was recorded with the green genotype, ~ threefold in the Red genotype and ~ fourfold in the Pony genotype. As a result, TFC values reached 18 mg GAE g^{-1} FW for the Green genotype, 20 mg GAE g^{-1} FW for the Red genotype and 29 mg GAE g^{-1} FW for the Pony genotype.

Salt Stress Induced Free Radical Scavenging Antioxidant Activity

In our study the free radical scavenging antioxidant activity was measured through the DPPH radical degradation method. The data showed that in absence of salt stress, the percentage of DPPH inhibition was around 38 to 39 (Fig. 3a) (Table 1). Interestingly salt stress significantly increased free radical scavenging antioxidant activity in the different genotypes. For instance, after treatment with 100 mM NaCl, the DPPH inhibition increased to 42% for both the Red and Pony genotype, and to 90%for the Green genotype. Moreover, after treatment with 200 mM, the DPPH inhibition increased to 60% for the Red genotype, 70% for the Pony genotype, and 90% for the Green genotype. Green genotype was found to have the highest free radical scavenging activity with increasing salinity. Hence, methanolic extracts of the Green genotype have a higher free radical neutralizing power than those of Red and Pony genotypes.

Moreover, the total antioxidant activity (TAA) in the leaves of the amaranth genotypes progressively increased

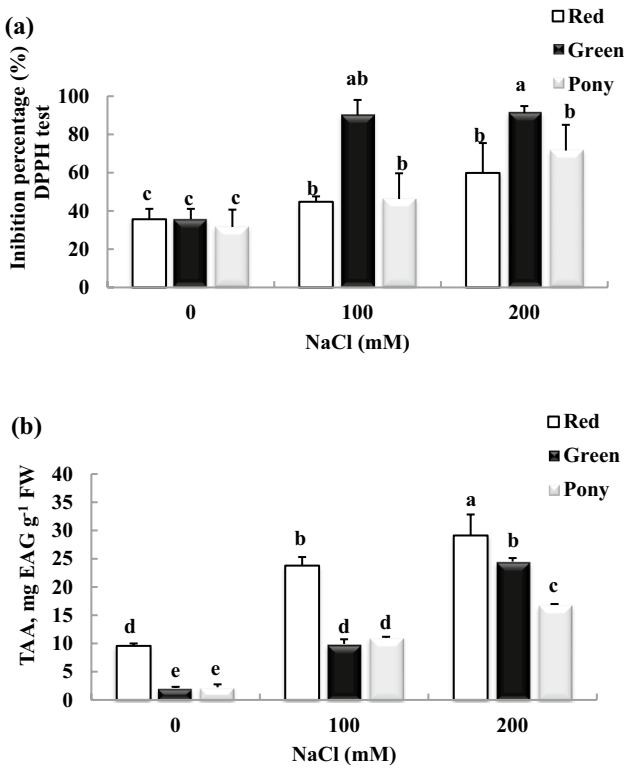

Fig. 3 Percentage of free radical inhibition (DPPH test) (**a**) and total antioxidant activity TAA (**b**) in three amaranth (*Amaranthus caudatus* L.) genotypes subjected to salt treatments (0, 100 and 200 mM NaCl). Data are means of 3 replicates ± SE and values followed by at least one letter are not statistically different at $p < 0.05$ according to Duncan's test

in response to the NaCl treatment (Fig. 3b). For instance, in the control condition, the TAA in the Red genotype was 10 mg EAG g^{-1} FW; it increased to 24 and 29 mg EAG g^{-1} FW under treatments with 100 and 200 mM NaCl, respectively. Likewise, in the Green and Pony genotypes, TAA increased from ~ 2 mg EAG g^{-1} FW in control condition to ~ 10 EAG g^{-1} FW under 100 mM NaCl, and to 25 and 17 mg EAG g^{-1} FW, respectively, under 200 mM NaCl treatment. Therefore, salt stress enhanced antioxidant activity in the stressed plants at the highest salt stress treatment, leaf antioxidant activities were the highest. In addition, Red genotype showed the highest total antioxidant activities among the other genotypes.

Salt Stress Increased Glutathione Content

Salt stress has an effect on the total glutathione cycle in amaranth. In the present work, NaCl treatment had a significant effect on GSH_T concentrations, and the increase was more pronounced in the Red genotype than in the Green and Pony genotypes (Fig. 4a) (Table 1). Specifically, Red genotype exhibited an increase of GSH_T concentration from 0.14 µmol g^{-1} FW in control condition to 0.22 and 0.27 µmol

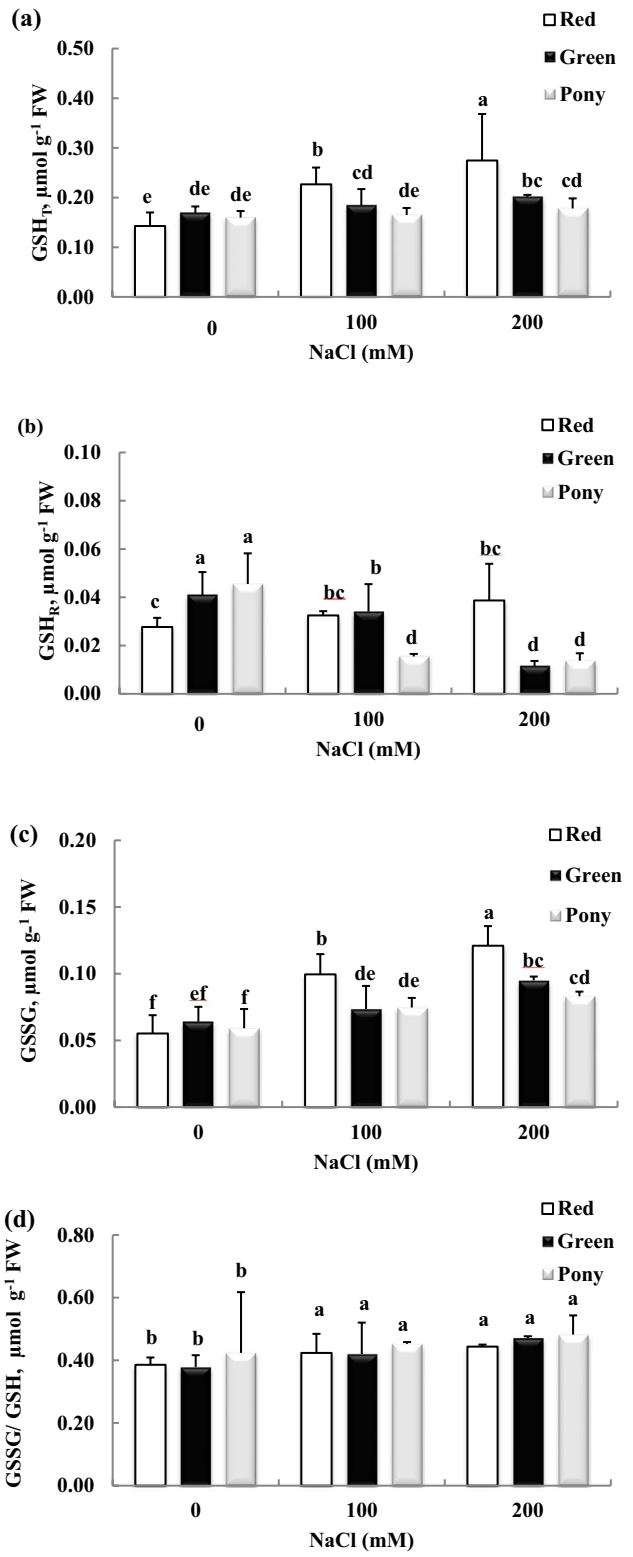

Fig. 4 Total glutathione content GSH_T (**a**), reduced glutathione content GSH_R (**b**), oxidized glutathione content (GSSG) (**c**) and GSSG/GSH ratio (**d**) in three amaranth (*Amaranthus caudatus* L.) genotypes subjected to salt treatments (0, 100 and 200 mM NaCl). Data are means of 3 replicates ± SE and values followed by at least one letter are not statistically different at $p < 0.05$ according to Duncan's test

g^{-1} FW under 100 and 200 mM NaCl treatments, respectively. In contrast, the Green and Pony genotypes maintained relatively stable GSH_T concentrations despite the increasing salinity treatment, compared with the control.

Also, on the one hand, there was a significant decrease in the concentrations of GSH_R with increasing salinity treatments (Fig. 4b) (Table 1). Indeed, a decrease of around 60 and 75% was noticed in Green and Pony genotypes at 100 and 200 mM NaCl compared to the control, respectively. On the other hand, an increase of 25% in Red genotype was noticed in response to 200 mM NaCl.

Furthermore, salinity significantly increased the concentration of GSSG (Fig. 4c) (Table 1). Such an increase was observed in all three genotypes but was more pronounced in the Red genotype. Indeed, an increase of ~ 0.045 and ~ 0.07 µmol g^{-1} FW was found in this latter genotype when treated with 100 and 200 mM NaCl, compared with the control, respectively. In contrast, the Green and Pony genotypes exhibited increases of only ~ 0.01 and 0.03 µmol g^{-1} FW when treated with 100 and 200 mM NaCl, compared to their respective controls, respectively. Regarding the GSH/GSSG ratio, salinity increased this ratio significantly and equally in all three genotypes (Fig. 4d). This indicates that despite the observed differences in the absolute concentrations of GSH and GSSG, the proportion between the two remained relatively constant with increasing salinity.

Salt Stress Decreased Tocopherol Content

Our results show that salinity significantly decreased the different α, β, γ and δ isoforms (Table 2). A treatment with 200 mM NaCl strongly decreased the levels of α tocopherol in Green and Pony genotypes, and a lower extend in Red genotype. Indeed, the decrease compared to their respective controls recorded was in the order of 42 and 73% in Green and Pony genotype, respectively, and only 29% in Red genotype. Total tocopherol levels decreased significantly with increasing salinity. Indeed, this decrease, compared to their respective control, was 80 and 45% in Green and Red genotypes, respectively; whereas in Pony genotype, it was only 15%.

Salt Stress Increased Membrane Dynamics

To investigate the early response of effects of amaranth cells to salt stress on their membrane dynamics, subcellular staining changes of the styryl dye FM4-64 were compared in root epidermis cells of seedlings of the three genotypes. Firstly, the mean gray value recorded in the cells, which indicates the intensity of dye uptake (and hence plasma membrane dynamics) by the endocytosis process, was monitored (Figs. 5a, 6). In control condition, the mean grey values of the three genotypes were at a basal level of ~ 400–900, whereas under salt stress, there was a significant increase to a level of ~ 1000–3000. Importantly, the highest increase was observed with Pony genotype (+ 91%), followed by Red genotype (+ 79%), and finally Green genotype (+ 57%); this indicates a variability in the response. Finally, the percentage of cells showing intracellular labeling was controlled to reflect labeled intracellular compartments (vesicles and other organites) labelled by the dye, and thus the intensity of dye penetration into the cell (Fig. 5b). This latter measurement indicates again the membrane dynamics upon salt stress. In control condition, the basal level of cells showing intracellular labelling was ~ 10% for the three genotypes. Importantly, under salt treatment, the levels increased to 77, 55, and 83% for Red, Green and Pony genotypes, respectively.

Table 2 Tocopherol α, β, γ and δ contents in three amaranth (*Amaranthus caudatus* L.) genotypes subjected to salt treatments (0, 100 and 200 mM NaCl)

NaCl	α (µg/g FW)	β (µg/g FW)	γ (µg/g FW)	δ (µg/g FW)	Total Tocopherol
Red					
Témoin	5.86 ± 2.55^{c}	0.00	2.58 ± 0.16^{b}	$1.70 \pm .46^{b}$	10.14 ± 2.54^{bc}
100 mM	6.58 ± 2.21^{bc}	0.00	1.53 ± 0.24^{cd}	1.22 ± 0.56^{c}	9.33 ± 3.00^{bc}
200 mM	4.18 ± 0.30^{d}	0.00	1.32 ± 0.25^{d}	0.16 ± 0.01^{e}	5.66 ± 0.54^{d}
Green					
Témoin	8.55 ± 3.86^{a}	0.21 ± 0.09^{b}	3.78 ± 1.99^{a}	2.40 ± 0.47^{a}	14.95 ± 0.6^{a}
100 mM	7.38 ± 0.22^{abc}	0.00	1.44 ± 0.08^{cd}	1.41 ± 0.10^{bc}	10.23 ± 0.2^{bc}
200 mM	2.35 ± 1.16^{e}	0.00	0.08 ± 0^{e}	0.79 ± 0.26^{d}	3.22 ± 1.31^{e}
Pony					
Témoin	6.85 ± 1.32^{bc}	0.42 ± 0.07^{a}	1.65 ± 0.09^{cd}	1.06 ± 0.30^{cd}	9.99 ± 2.13^{bc}
100 mM	7.83 ± 1.24^{ab}	0.41 ± 0.09^{a}	1.90 ± 0.30^{cd}	1.14 ± 0.14^{cd}	11.28 ± 2.40^{b}
200 mM	3.95 ± 1.26^{d}	0.42 ± 0.28^{b}	1.98 ± 0.98^{c}	2.29 ± 1.12^{a}	8.49 ± 2.34^{c}

Data are means of 3 replicates ± SE and values followed by at least one letter are not statistically different at $p < 0.05$ according to Duncan's test

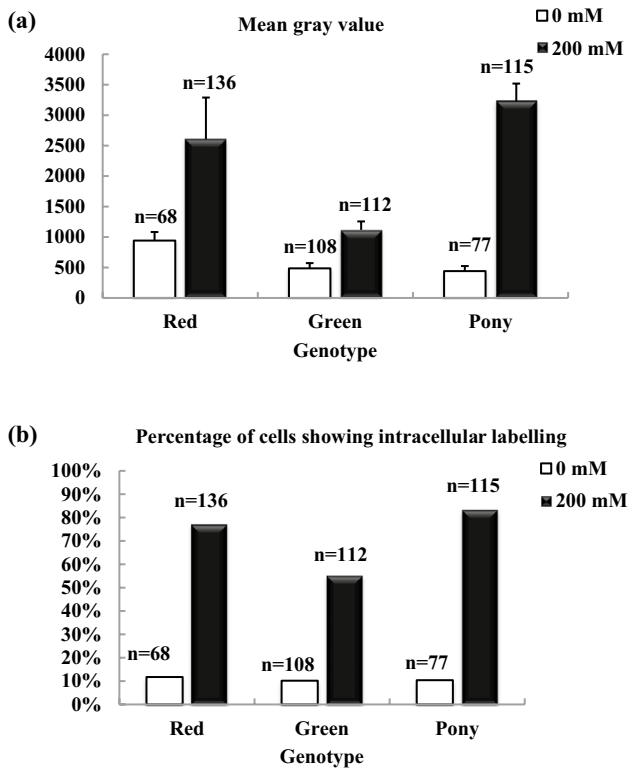

Fig. 5 Quantification of *A. caudatus* L. epidermal-root-cell labelling by styryl dye FM 4–64 under salt treatments. Mean gray value (**a**) and the percentage of cells showing intracellular labelling (**b**) are shown for the three genotypes under control (white bars) or 200 mM NaCl treatment (black bars). The numbers of cells analyzed are indicated (n)

Discussion

To improve crop growth and productivity in stressful environments, it is important to understand the mechanisms that enable plants to tolerate stress. Salinity significantly affects plant growth by altering physiological and biochemical traits. It is therefore important and mandatory to use suitable techniques to study the physiological, biochemical and molecular responses of plants to salt treatment (Gupta and Huang 2014).

Leaf plant cell plasma membranes are essential components of plant cells, providing protection and helping to maintain cellular homeostasis. The main constituents of plant membranes are glycerolipids, glycerophospholipids, and lipid sterols, between which proteins can be inserted, as described in the mosaic model (Chen et al. 2019). These lipid components contribute to the integrity and functionality of plant cell membranes. The peroxidation of membrane lipids, especially phospholipid the major fatty acid of plant cell plasma membrane, serves as an indicator of oxidative damage caused by environmental stress. In the present study, the three genotypes showed a similar degree of lipid peroxidation with the high salinity treatment (200 mM NaCl), which can be explained by a similarity in the composition of the lipid fractions, in particular the fatty acid composition. It has been reported that the increasing of malondialdehyde (MDA) concentrations under salinity stress indicate the degree of damage encountered by the plasma cell membrane. Venskutonis and Kraujalis (2013) reported that composition by the fatty acid of *A. caudatus* is particularly high in unsaturated fatty acids, accounting for approximately 73% of the total fatty acid content. In the leaves the main components are linoleic acid (44.5–47.8%) and oleic acid (23.7–28.8%), according to Alvarez-Jubete et al. (2010). According to Ahammed et al. (2018), MDA is the end product of lipid peroxidation under salt stress and indicate oxidative stress where reactive oxygen species (ROS), produced by salinity stress, induced damage to the lipid plasma membrane. Our findings contradict those of Estrada et al. (2021), where a treatment with 100 mM NaCl didn't induce a change in MDA content in amaranth, but in quinoa, MDA content increased significantly under such stress conditions. With regard to MDA accumulation, it is necessary to keep the idea that MDA may act as a protective mechanism rather than an indicator of oxidative damage, as MDA may exert a positive role by activating regulatory genes involved in oxidative homeostasis (Morales and Munné-Bosch 2019). This could occur in quinoa leaves where MDA content was twice as high in salt as in control conditions. Morales and Munné-Bosch (2019) reported that the accumulation of MDA can be considered as a protective mechanism since it activates various regulatory genes involved in oxidative homeostasis.

Secondary metabolites such as polyphenols, flavonoids and others have important functions in signaling between different parts of the plant. They also aid in the mobilization of nutrients, ensuring that the plant receives the necessary resources for growth and development (Sharma et al. 2019). The present study showed that Amaranth leaves responded by accumulating phenolic and flavonoid compounds, known for their antioxidant properties, to overcome the harmful effects of reactive oxygen species (ROS) generated during salt treatment. Similarly to our study it has been reported that salinity stress increase the TPC in leaves of *Plantago ovata* (Golkar et al. 2017), safflower (Golkar and Taghizadeh 2018) and groundnut (Mohan and Shashidharan 2019). It has indicated that, salinity stress stimulates the biosynthesis of phenolic compounds which depends on the species and the treatment of NaCl (Bettaieb Rebey et al. 2017). This accumulation suggests that plants activate the phenylpropanoid biosynthetic pathway to counteract oxidative stress. Indeed, this mechanism can be explained by the role of polyphenols as antioxidants, which play a crucial role in attenuating the impact of ROS instead of catalase, in order to mitigate the harmful effect of ROS inside plant cells. In this context, Chen et al. (2019) reported that phenolic compounds are

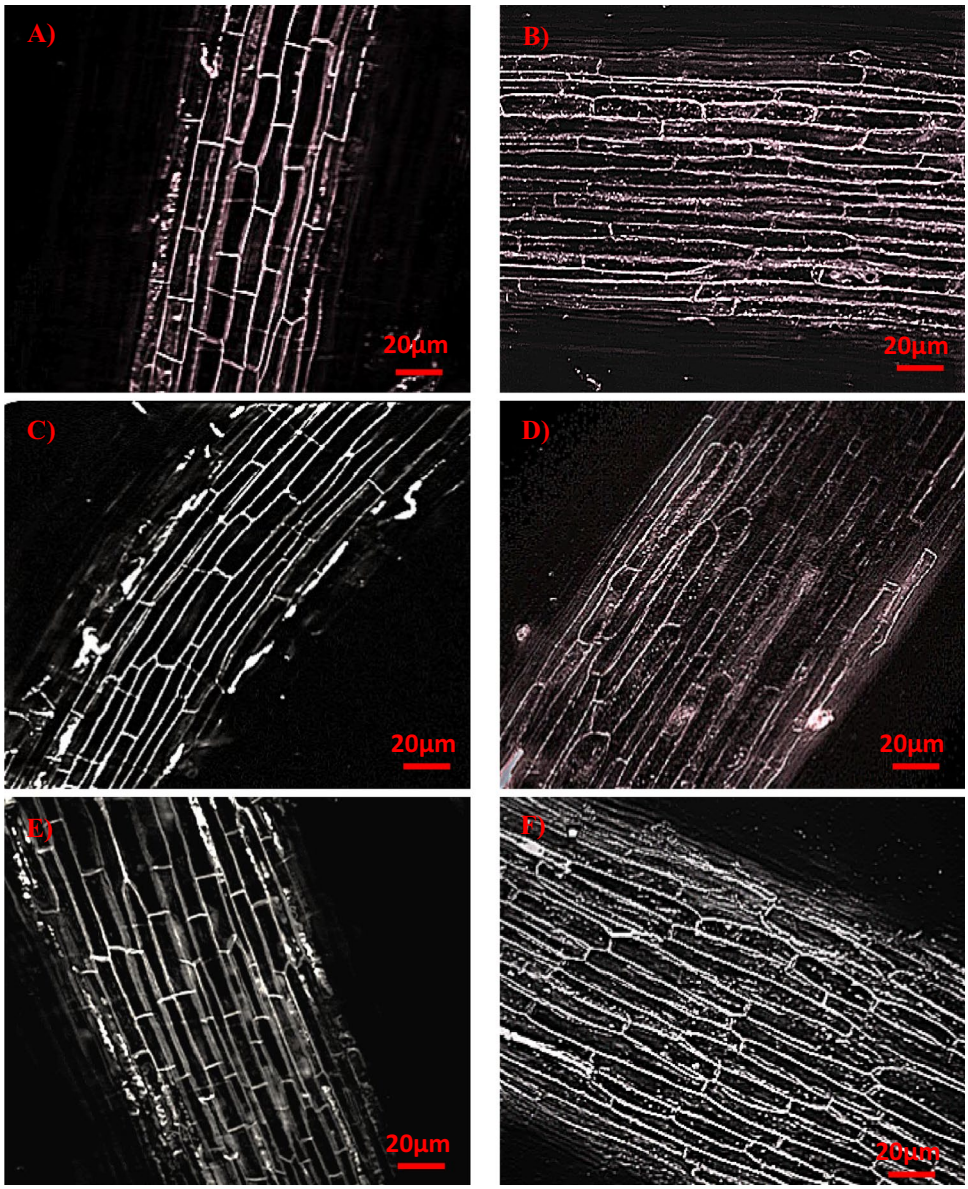

Fig. 6 Microscopic images of FM4-64-stained epidermal cells in the elongation zone of a root: Red: **A** control, **B** treated with 200 mM NaCl for 10 min, Green: **C** control, **D** treated with 200 mM NaCl and Pony: **E** control, **F** treated with 200 mM NaCl for 10 min

known for their powerful antioxidant properties and play a crucial role in scavenging these harmful ROS when plants are subjected to salt stress. The results of antioxidant assessments using ABTS$^{\circ+}$, β-carotene bleaching, and DPPH$^{\circ}$-scavenging techniques were highly consistent with the presence of TPC, as highlighted by Gorinstein et al. (2007). On the other hand, due to their high antioxidant potential, abiotic stress stimulates the biosynthesis of flavonoids in higher concentrations to modulate the antioxidant system against oxidative stress in plants. (Arbona et al. 2013). They act as non-enzymatic antioxidants to mitigate the effect of ROS (Syvertsen and Garcia-Sanchez 2014). The present study showed that TFC was highest in Pony genotype, followed by Red and finally Green. In comparison with the work carried out by (Tebini et al. 2022a), a low level of catalase enzyme activity was reported in leaves stressed with 200 mM NaCl, which may be explained by the activation of genes that condense falavonoid biosynthesis to provide protection against ROS damage. It has been reported that, certain genes such as *VvbHLH1* coding for flavonoid biosynthesis in *Arabidopsis thaliana* are involved in increasing the production of flavonoids by controlling the genes responsible for the biosynthetic pathways (Wang et al. 2016). These genes also allow plants to cope with high salt levels (Golkar and Taghizadeh 2018).

Glutathione is a crucial metabolite in the antioxidant defense system of plants. Among its functions are diverse and include influencing seedling growth (Hussain et al. 2009), reducing the influx of sodium ions by stabilizing the cell membrane, and mitigating oxidative stress by decreasing

lipid peroxidation. In the current study, the leaves GSH content increased in all three Amaranth genotypes under different salinity treatments. Red genotype showed a greater increase in GSH than the genotypes Green and Pony. In comparison, the study carried out by Tebini et al. (2022a) showed a decrease in the activity of antioxidant enzymes in leaves with a NaCl concentration of 200 mM, i.e. APX, GPOX and CAT, indicating that the glutathione in Red is used as an antioxidant against the harmful effects of ROS. Interestingly, the increase in total glutathione suggests a strategy for plants to overcome oxidative stress. However, the decrease in reduced glutathione indicates a change in the redox balance under salt stress conditions, leading to a more oxidizing cellular environment. The increase in oxidized glutathione also confirms the idea of this change. In the same context, Mittal et al. (2020) reported that glutathione, as a signaling molecule, contributes to maintaining cellular redox balance, and its increased accumulation contributes to osmotic adjustment, improving salt tolerance of plants. Similar research conducted on wheat demonstrated that GSH enhances cell viability under salinity stress (Ahanger et al. 2019). Additionally, in plant GSH aids in the detoxification of methylglyoxal by enhancing the activity of glyoxalase enzymes in the glyoxalase system, leading to improved salinity tolerance (Rehman et al. 2021).

In contrast to glutathione, the present study founded that salinity treatment reduced significantly tocopherols content in amaranth leaves, which are lipophilic powerful antioxidant compound protecting plants from oxidative damage especially ROS. Among the four commonly known lipophilic isomers of tocopherol (α, β, γ, and δ), α-tocopherol is recognized as one of the most active antioxidants (Das and Roychoudhury 2014). Regardless of the specific tocopherol type, these compounds play a critical role in neutralizing lipid peroxy radicals, oxygen free radicals, and singlet oxygen (Zandi and Schnug 2022). The result showed that all four isoforms (α, β, γ, and δ) were detected in the Pony genotype, both in the presence and absence of NaCl, suggesting their involvement in the antioxidant defense system. In the Green genotype, the β-tocopherol isoform is present only in control conditions and absent in the presence of NaCl, while in the Red genotype, it is absent altogether. In contrast to our results for the two genotypes Red and Green, it has been reported that β-tocopherol is present in remarkably higher concentrations in *A. cruentus* (Venskutonis and Kraujalis 2013). Skłodowska et al. (2009) conducted an experiment showing that increasing the concentration of tocopherol improves the tolerance level of tomato plants subjected to different salinity stress treatment. Similarly, a study by Mostafa et al. (2015) on *Glycine max* treated with different concentrations of NaCl demonstrated that foliar application of tocopherol improves photosynthesis and plant growth.. Tocopherols also have the ability to eliminate lipid peroxides and oxygen radicals, as well as deactivating singlet oxygen. (Mushtaq et al. 2020). This is in agreement with the findings of the current study, where the decrease in tocopherol content is significantly correlated with an increase in lipid membrane peroxides (MDA). They are involved in mitigating lipid peroxidation and may interact with the signaling pathways that mediate abiotic and biotic signals (Bafeel and Ibrahim 2008).

Vesicular trafficking is a vital process within cells that involves the movement of membrane-enclosed substances. It plays a crucial role in the proper functioning of cells. One of the main routes in this process involves the transport of vesicles containing newly synthesized materials from the endoplasmic reticulum (ER) to the Golgi apparatus, and from there to either the cell outer membrane (plasma membrane) or to specialized compartments called vacuoles. This movement in the forward direction is known as anterograde biosynthetic trafficking (Baral et al. 2015). Additionally, there is also a documented process of retrograde trafficking, where cargo is transported from the Golgi back to the ER. Another aspect of vesicular trafficking is the formation of vesicles through the inward folding of the plasma membrane, which is referred to as endocytosis. Endocytosed cargo follows various intermediate stages and is ultimately directed either to vacuoles for degradation or recycled back to the plasma membrane. Styryl dye FM4-64 was used, which immediately marks the plasma membrane, as it is inserted and anchored in the outer leaflet of the plasma membrane lipid bilayer (Fischer-Parton et al. 2000). With time, FM4-64 is internalized by the endocytosis and due to vesicular trafficking, the dye labels several intracellular compartments. Thus, FM4-64 has been considered as a reference tool to investigate membrane dynamics. Here, as early as ~ 10 min after the onset of salt stress, a significant increase of intracellular labelling by FM4-64 upon salt treatment, suggesting an increase in membrane dynamics. This series of results questioned the role of membrane dynamics in the establishment of oxidative stress response. Hence, two stimuli (salicylic acid and salt) induce ROS production and internalization of water channel Plasma membrane Intrinsic Proteins (PIPs) (Boursiac et al. 2008).Also, salt stress accelerates the recycling of PIPs between the plasma membrane and the Trans Golgi Network (Luu et al. 2012). In the absence of salt stress, the PIP recycling pathway was found to involve clathrin in endocytosis. However, under the stress, the endocytosis process would be independent of clathrin (Luu et al. 2012). Dynamics of PIPs reflecting membrane dynamics, this huge reorganization of membranes in the cell might be a key step in the establishment of response to oxidative stress.

In conclusion, the present work demonstrated that salt stress induced an increase in membrane dynamics which might reflects important mechanisms in the establishment of oxidative stress. Understanding these responses may help

develop strategies to improve salt tolerance in amaranth and other crops, which is crucial given the growing challenges of soil salinization and climate change.

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Declarations

Conflict of Interest The authors declare no competing financial interest.

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