Lack of mitochondrial thioredoxin *o*1 is compensated by antioxidant components under salinity in *Arabidopsis thaliana* plants

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In a changing environment, plants are able to acclimate to the new conditions by regulating their metabolism through the antioxidant and redox systems involved in the stress response. Here we studied a mitochondrial thioredoxin in wild type (WT) *Arabidopis thaliana* and two *Attrxo1* mutant lines grown in the absence or presence of 100 mM NaCl. Compared to WT plants, no evident phenotype was observed in the mutant plants in control condition, although they had higher number of stomata, loss of water, nitric oxide and carbonyl protein contents as well as higher activity of superoxide dismutase (SOD) and catalase enzymes than WT plants. Under salinity, the mutants presented lower water loss and higher stomatal closure, H_2O_2 and lipid peroxidation levels accompanied by higher enzymatic activity of catalase and the different SOD isoenzymes compared to WT plants. These inductions may collaborate in the maintenance of plant integrity and growth observed under saline conditions, possibly as a way to compensate the lack of *TRXo1*. We discuss the potential of TRX*o*1 to influence the development of the whole plant under saline conditions, which have great value for the agronomy of plants growing under unfavourable environment.

Abbreviations − AOX, alternative oxidase; APX, ascorbate peroxidase; ASC, ascorbate; CAT, Catalase; DHAR, dehydroascorbate reductase; ETC, electron transport chain; FW, fresh weight; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; KO, knockout *Attrxo1* mutant; MDHAR, monodehydroascorbate reductase; Mn-SOD, manganese superoxide dismutase; NO, nitric oxide; PCNA, proliferating cellular nuclear antigen; POX, peroxidase; PRX, peroxiredoxin; ROS, reactive oxygen species; TRX, thioredoxin; WT, wild type.

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Introduction

Salinity is an important ecological factor influencing all kinds of organisms including crops for which the yield is usually decreased. Physiological and molecular aspects of plants under salt stress have been extensively studied in terms of its effects on plant growth, stomatal conductance, water relation, ion metabolism, photosynthesis, respiration and oxidative stress generation (Camejo et al. 2013, Flexas et al. 2004, Martí et al. 2009). Under salt stress, as well as in other unfavourable situations, the production of reactive oxygen species (ROS) occurs, which could have negative consequences depending on the intensity and duration of the stress, affecting cellular structures, redox balance and metabolism. However, ROS can also have positive implications due to the signaling nature of some of the species produced (Baxter et al. 2014, Foyer and Noctor 2013). In plants, ROS production is kept under tight control by an efficient antioxidative system, which includes both enzymatic and non-enzymatic compounds, modulating intracellular ROS concentration and in this way setting the cellular redox homeostasis. Together with chloroplasts and peroxisomes, mitochondria account for a significant proportion of the total plant ROS generation (Noctor et al. 2007, Sevilla et al. 2015a). In this organelle, the radical superoxide ($O_2^{\cdot-}$) generated by the electron transport chain (ETC) is mainly converted to H_2O_2 by manganese superoxide dismutase (Mn-SOD, Sevilla et al. 1982) and it is decomposed by the mitochondrial peroxidase activities dependent on the following antioxidants (1) ascorbate (ASC) for the hemo-containing enzyme ascorbate peroxidase (APX), (2) the thiol reductant glutathione (GSH) for the glutathione reductase and peroxidase (GR, GPX) and (3) the thioredoxin (TRX)/peroxiredoxin (PRX) system (reviewed by Moller 2001, Lázaro et al. 2013). The generated oxidized forms of ASC are then reduced by NAD(P)H-dependent monodehydroascorbate reductase (MDHAR) and GSH-dependent dehydroascorbate reductase (DHAR), while oxidized GSSG is reduced by the flavoprotein glutathione reductase (GR) in an NADPH-dependent manner (Jiménez et al. 1997). TRXs are small proteins containing two redox-active Cys residues in a highly conserved redox-active site (Holmgren 1989). They are involved in a variety of cellular processes like the regulation of redox status of so-called target proteins through thiol-disulfide exchange reactions (Martí et al. 2009, Zhang et al. 2011). In *Arabidopsis thaliana* (Arabidopsis), according to the amino acid sequence homology, TRXs can be divided into six main groups with different cellular locations: TRX*h*, TRX*f*, TRX*m*, TRX*x*, TRX*y* and TRX*o*. The presence of TRX in mitochondria (AtTRX*o*) was first reported by Laloi et al (2001) in Arabidopsis together with NADPH-TRX reductase (Reichheld et al. 2005), although a cytosolic TRX*h* was also reported to be located in this organelle in poplar (Gelhaye et al 2004) and several mitochondrial TRX targets from pea, spinach and potato tubers were reported using spinach TRX*m* and poplar TRX*h* as a bait (Balmer et al. 2004). A pea TRX*o*1 was later described to be present in mitochondria and nuclei where it regulates several processes as respiration and cell cycle progression through its interaction with target proteins as alternative oxidase (AOX) or proliferating cellular nuclear antigen (PCNA) among others (Martí et al. 2009, Calderón et al. 2017).

In mitochondria, a strong interaction occurs between PsTRX*o*1 and peroxiredoxin IIF (PsPRXIIF) which together with sulfiredoxin, may constitute the mitochondria redox system (Barranco-Medina et al. 2008, Iglesias-Baena et al. 2011). Further analysis showed that additional mitochondrial proteins are targets of TRX*o*1, suggesting that it may also control their redox status (Balmer et al. 2015). In fact, Yoshida et al. (2013) reported that a large number of TRX*o* target candidates in plant mitochondria are linked to multiple biological processes, implying a broad impact on mitochondrial activity through the TRX system. The possible role of TRX*o* in the response of plants to abiotic stress, including salinity, and its involvement in plant tolerance to stress has been less studied (Barranco-Medina et al. 2007, Martí et al. 2011, Sevilla et al. 2015b), although its involvement in signal transduction has been proposed (Rouhier and Jaquot 2005).

Mitochondrial antioxidant and redox systems seem to be key players involved in the whole plant tolerance to stress. During salinity, an enhanced tolerance of transgenic Arabidopsis plants overexpressing mitochondrial manganese superoxide dismutase (Mn-SOD) has been reported (Wang et al. 2004). Moreover, more than half of the 300 salt stress-induced genes in Arabidopsis are predicted to code for mitochondrial proteins (Heazlewood et al. 2007). We have previously reported that mitochondrial TRX*o* could provide the cell with a mechanism by which it can respond to a changing environment through the modulation of the activity of its target enzymes and probably also protecting mitochondria from oxidative stress together with Mn-SOD, AOX, and PRXII F (Martí et al. 2011). The increase in the oxidoreductase activity of TRX did not parallel the increase in oxidized proteins and lipids in mitochondria under extended NaCl stress (14 days at 150 mM), whereas the impairment of the TRX function by a reduction of its activity during a shorter treatment (5 days) enhanced the susceptibility of the plants to stress-induced oxidation. Consequently, TRX may participate in the repair of oxidized proteins during environmental constraints (Martí et al. 2011).

To gain more insight into the effect of the lack of mitochondrial TRX*o*1, we studied the response of two Arabidopsis T-DNA mutant variants under salt stress. Changes in the enzymatic and non-enzymatic antioxidant systems Mn-SOD, CAT and the enzymes involved in the ascorbate-glutathione cycle APX, MDHAR, DHAR, GR as well as the antioxidants ASC and GSH were measured in WT and two *trxo1* mutants grown in the absence or presence of 100 mM NaCl for 7 days. Simultaneously, several physiological parameters of growth and inorganic ion accumulation were also evaluated. The biochemical and physiological characterization of Arabidopsis mutants unravelled the biological significance of the mitochondrial TRX*o* in response to salt stress.

Materials and methods

Plant material and growth conditions

Seeds of *Arabidopsis thaliana* L. wild-type (ecotype Columbia) and two T-DNA insertion lines in Columbia knocking out *AtTRXo1* (At2g35010) namely SALK_143294C (line 590, named KO-1) and SALK_042792 (line 374, named KO-2) were obtained from the European Arabidopsis Stock Centre

(NASC; http://arabidopsis.info/; Nottingham University, UK). The homozygous plants were selected and the lack of *AtTRXo1* expression was checked in germinating seeds as described in Ortiz-Espín et al. (2017).

Plants (1 plant per pot) were grown in substrate containing perlite:peat soil (1:3 v:v) under controlled conditions of light (150 µmol m^{-2} s^{-1} photosynthetic active radiation), photopheriod (16 h light/8 h dark), relative humidity (60%) and temperature (23/18ºC light/dark). Fourteen day-old plants were either exposed to 100 mM NaCl for 7 days or left without NaCl for 7 days before being harvested.

Growth parameters and nutrient status

The effect of salt on plant growth was estimated measuring fresh and dry weight, rosette diameter, number of leaves and leaf width. Oven-dried leaf tissues were digested in a microwave (Ethos 1, Milestone, Sorisole, Italy) with HNO_3:H_2O_2 (4:1), and the mineral concentration was determined by inductively coupled plasma spectrometry (ICP) (Iris Intrepid II; Thermo Electron Corporation, Franklin, MA).

Water loss and stomatal conductance

Detached leaves from 3-week-old plants were collected and weighed at various time intervals, and the loss of fresh weight (%) was used to indicate water loss. The experiment was conducted on the laboratory bench at 55% relative humidity and four replicates of five fully expanded leaves for each line and treatment were measured (Li et al. 2013).

Stomatal conductance (g_s) was measured with an open infrared gas-exchange analyser system (Li-6400; Li-Cor Inc., Lincoln, NE) as described in León-Sánchez et al. (2016). Six measurements on a fully expanded leaf from six different plants of each genotype were carried out in untreated and salt-treated 21-days old plants at 6–8 h of the light period under a light-saturating photon flux density (PFD) of 1000 mol m^{-2} s^{-1} (provided by the light source of the Li-6400 with 10% blue light).

Measurement and analysis of stomatal density and aperture

Nail polish imprints were taken from the abaxial surface of fully developed leaves from WT and KO-*trxo1* mutant plants as described by Schlüter et al. (2003). Stomatal densities were determined by light microscopy from leaf imprints of five individual plants and four independent counts were carried out on each leaf (n>400 stomata per genotype and treatment).

To examine the stomatal movement, fully expanded leaves were detached from plants in the first hour of the light period (with the majority of stomata closed, data not shown), and floated with the

abaxial side turned down in a Petri dish containing MES/KOH buffer (5 mM KCl, 10 mM MES (2-(N-morpholino) ethanesulfonic acid), 50 μM $CaCl_2$, pH 6.15) for 2 h under constant cool light (100-120 μmol m^{-2} s^{-1} PAR) at 23ºC (Eisele et al. 2016). Imaging of stomata was performed with a light microscope (Olympus BX40, Tokyo, Japan) equipped with a CCD camera and the image analysis was performed using ImageJ software (https://imagej.nih.gov/ij/). The stomatal aperture index (SAI) was calculated by division of the stomatal aperture width through the length. At least 25 stomata per leaf were analysed and four leaves per each genotype and treatment were used for statistical analysis.

Hydrogen peroxide, lipid peroxidation, carbonyl proteins and nitric oxide contents

The H_2O_2 content was measured by eFOX (ethanol ferrous oxidation−xylenol orange) method according to Cheeseman (2006). Fresh tissue (0.1 g of leaf) was immediately submerged in 1 ml acetone acidified with 25 mM H_2SO_4 and frozen in liquid nitrogen. Samples were processed as described by Camejo et al. (2011). The method is based on the peroxide-mediated oxidation of Fe^{2+}, followed by the reaction of Fe^{3+} with xylenol orange (*o*-cresolsulfonephthalein-3´,3´´-bis[methylimino] diacetic acid tetrasodium salt, Sigma-Aldrich, St. Louis, MO), which was detected after 45 min. The amount of H_2O_2 was determined based on the difference in absorption at 550 and 850 nm using a standard curve (0-200 μM).

The extent of lipid peroxidation was estimated by determining the concentration of substances reacting with thiobarbituric acid according to Buege and Aust (1978) with minor modifications. Samples were incubated with a mixture reaction containing 15 % (w/v) trichloroacetic acid, 0.37 % (w/v) thiobarbituric acid, 0.01 % (w/v) butylhydroxytoluene and 0.25 M HCl at 95ºC for 15 min and then were centrifuged at 2 000 *g* for 5 min. Supernatants were collected and the absorbance change at 535 nm was measured.

Carbonyl protein content was measured as carbonyl content according to Levine et al. (1990) using 2,4-dinitrophenylhydrazine (DNPH) as described by Ortiz-Espín et al. (2015). The total soluble protein content was quantified using Bradford's method (Bradford 1976).

Nitric oxide (NO) content was analysed by fluorimetry using 4,5-Diaminofluorescein (DAF-2) as described by Camejo et al. (2013), measuring on a Shimadzu RF-5301PC spectrofluorometer (Tokyo, Japan) calibrated for excitation at 495 nm and emission 515 nm.

Leaf enzyme extraction

Leaf samples were homogenized in a mortar using 50 mM potassium phosphate buffer (pH 8.0) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM cysteine, 0.2% (v/v) Triton X-100, 1% (w/v) soluble polyvinylpyrrolidone and 0.1 mM phenylmethylsulphonylfluoride (PMSF). For APX extraction, 20 mM ascorbic acid was included in the extraction buffer and EDTA was omitted. The

homogenate was centrifuged at 15 000 *g* for 10 min, while the supernatant was immediately filtered through a Sephadex G 50 PD10 column (Pharmacia Biotech AB, Barcelona, Spain) equilibrated with the same buffer as used for homogenization. All operations were performed at 4°C.

SOD and catalase activities

Superoxide dismutase (SOD) (EC 1.15.1.1) activity was assayed according to Jiménez et al. (1997), following the ferricytochrome *c* reduction using xanthine/xanthine oxidase as the source of superoxide radicals (McCord and Fridovich 1969). One SOD unit (U) was defined as the quantity of enzyme that produces a 50% inhibition of the reduction of cytochrome *c*. Electrophoretic separation of the protein was performed by non-denaturing polyacrylamide gel electrophoresis (PAGE). Equal amounts of proteins were prefocused at 15 mA for 15 min, and then focused at 45 mA for 3 h. After electrophoresis, a photochemical method was used to visualize SOD activity (Beauchamp and Fridovich 1971). Isoenzyme identification was performed by selective inhibition with 2 mM KCN and 5 mM H_2O_2. The isoenzyme activities were quantified on an image analyzer (Gen Tools; Syngene, Cambridge, England).

Catalase (CAT; EC 1.11.1.6) activity was determined according to Aebi (1984) measuring the decrease in A_{240} due to the disappearance of H_2O_2 (ε=39.58 M^{-1} cm^{-1}).

Glutathione and ascorbate determinations

Tissue extraction was performed as described by Camejo et al. (2010) with some modifications. Frozen plant tissue (500 mg) was ground in liquid N_2 and the dry powder homogenized with 1 ml of cold (4°C) extraction solution (5% m-phosphoric acid (w/v) and 1 mM EDTA in 0.1% formic acid), supplemented with 1% polyvinyl-polypyrrolidone (w/v) just before use. Stock solutions containing 100 µM GSH, GSSG, ASC, DHA and GSNO were prepared in the same cold extraction solution. Analyses were carried out with an Agilent Ion Trap XCT Plus Mass Spectrometer (Agilent Technologies, Santa Clara, CA) with an electrospray ionization source (ESI) and coupled to an Agilent 1100 Series HPLC system (Agilent Technologies, Santa Clara, CA).

Ascorbate-glutathione cycle enzymes

Ascorbate peroxidase (APX; EC 1.11.1.11) activity was assayed according to Jiménez et al. (1997), measuring the oxidation rate of ASC between 1 and 60 s after starting the reaction by the addition of H_2O_2 (ε=2.8 mM^{-1} cm^{-1}).

Monodehydroascorbate reductase (MDHAR; EC 1.1.5.4) activity was assayed by the decrease in absorbance (A_{340}) due to the NADH oxidation (ε=6.22 mM^{-1} cm^{-1}; Jiménez et al. 1997). Monodehydroascorbate (MDHA) was generated by the ASC/ascorbate oxidase system. To determine the MDHAR activity, the rate of MDHA-independent NADH oxidation (without ASC and ascorbate

oxidase) was subtracted from the initial MDHA-dependent NADH oxidation rate (with ASC and ascorbate oxidase).

Dehydroascorbate reductase (DHAR; EC 1.8.5.1) activity was determined as described in Jiménez et al. (1997) by following the increase in A_{265} nm due to ASC formation using N_2-bubbled buffer (ε=14 $mM^{-1}cm^{-1}$). The reaction rate was corrected for the non-enzymatic reduction of dehydroascorbate (DHA) by reduced glutathione (GSH).

Glutathione reductase (GR; EC 1.6.4.2) activity was determined according to Foyer and Halliwell (1976). The reaction mixture contained 25 mM sodium phosphate buffer (pH 7.8), 0.2 mM EDTA, 0.5 mM oxidized glutathione (GSSG), 0.12 mM NADPH, and 0.1 ml enzyme extract. The NADPH oxidation was monitored by the decrease in the absorbance at 340 nm and the assay was performed at 25℃ for 2 min.

Statistical analysis

The experiments were conducted in a completely randomized design. Each experiment was repeated at least three times with three replicates per treatment for each genotype and at least four plants per replica. Data were subjected to two different statistical analysis: one for differences among genotypes in each condition (capital letters for control and lower-case letters for saline conditions) with an analysis of the variance (ANOVA, one factor) using the Tukey's test (P<0.05), and another test for the salt effect in each genotype (asterisk when significant differences under salinity compared with control condition) using the *t*-Student test (P<0.05). IBM SPSS Statistics 20 (Statistical Package for Social Sciences, 2011) programme was used for the data analysis.

Results

Growth parameters and nutrient status

After 21 days of growth in the presence of 100 mM NaCl, none of the mutant plants revealed any appreciable phenotype neither in control nor under salinity conditions when compared to WT plants (Fig. 1A) while a visible reduction of the growth was visible under salt stress for all the lines analysed. Rosette diameter (Fig. 1B) and leaf width (Fig. 1C) decreased similarly salt treated WT and *trxo1* mutant plants while no significant changes in the number of leaves were observed (Fig. 1D) in any of the genotypes.

Under control conditions, the KO-1 mutant contained lower Ca, P and Mg content while KO-2 contained lower K, P, Mg, S and Zn compared to WT (Table 1). Under 100 mM NaCl stress conditions, a higher content of Na was observed in the WT plants although remarkably, salinity did not increase significantly the Na content in the KO-2 mutant. Under this condition, K and S decreased in all the genotypes. Increases in Mg, Zn and Mn occurred only in the two *trxo1* mutant lines.

No statistical differences in fresh (Fig. 2A) and dry (Fig. 2B) weights were found between WT and *trxo1* mutants in control or saline conditions, although when plants were exposed to the salt treatment, plant biomass significantly decreased in all the genotypes (around 25-30%).

Stomatal conductance and water loss

Stomatal conductance (g_s) did not present significant differences between WT and *trxo1* fully expanded leaves in control condition and decreased similarly in all genotypes under salinity (Fig. 3A). When these leaves were subjected to a dehydration assay measuring the percentage of weight loss, significant differences were found in control conditions for KO-2 line which had a higher loss of water (around 50%) than WT and KO-1 plants (between 20-30% respectively) (Fig. 3B). However, under salinity, the two KO lines retained more water than WT plants, KO-1 being the line with the highest values of leaf weight during the 120 min measured (Fig. 3C).

Stomatal density and aperture

Stomatal visualization and aperture were done on epidermal peels of the WT and *trxo1* lines after induction of aperture, measuring the ratio of width over length of the pore. No visual changes were observed in any of the genotypes in both conditions (Fig. 4A). Interestingly, the number of stomata per foliar area was found higher in the two KO lines than in WT plants mainly in control and to a lesser extend under saline conditions (Fig. 4B). The stomatal index indicated that compared to WT plants, the two mutant lines presented a reduced stomatal aperture in both control and saline conditions (Fig. 4C).

Nitric oxide, hydrogen peroxide and oxidative status

Both *trxo1* lines contained higher levels of NO than WT plants under control conditions while only KO-1 line showed higher level than WT under salinity condition. A significant decrease in NO content with salt was only found in the KO-1 line (Fig. 5A). For H_2O_2 content, no differences between WT and *trxo1* mutant lines were found in control conditions (Fig. 5B). Under salinity the H_2O_2 content increased in all the genotypes and differences were observed in the *trxo1* mutant lines which showed a higher increase (about 1.5-fold) than WT plants.

The analysis of parameters indicative of oxidative stress revealed that the lipid peroxidation measured as malondialdehyde content was no significantly different among genotypes in control conditions (Fig. 5C), while an increased level of carbonyl (CO) protein groups was found in the *trxo1* mutants (Fig. 5D). However, salt-treatment increased both oxidative parameters in all the genotypes and the MDA level was higher in the *trxo1* mutant lines than in WT plants, while protein oxidation was similar (Fig. 5C, D).

Glutathione, *S*-nitrosoglutathione and ascorbate content

Under control conditions no significant differences were found between WT and *trxo1* mutants in the level of GSH, GSSG, GSNO or ASC (except KO-2 with a higher GSNO content) (Fig. 6A-D). Under salinity, differences were only found in the KO-1 line with a decrease in GSH, GSSG and ASC contents. The redox state of glutathione was similar for all the genotypes in both conditions except a decrease observed in the KO-2 mutant under control conditions (Fig. 6E).

TRX*o*1 mutant effect on antioxidant enzymes

Analysis of the total superoxide dismutase (SOD) activity in WT and *trxo1* mutant plants revealed significant differences under control and saline conditions with higher values in both mutants (Fig. 7A). With the salt treatment, all genotypes presented an increased activity. When the activity of the different SOD isoenzymes was visualized on polyacrylamide gels after electrophoresis, the quantification of the bands revealed that Mn-SOD, Fe-SOD and Cu/Zn-SOD activities were higher in the *trxo1* mutant plants than WT under both conditions (Fig. 7B) and that the activities increased with the salinity stress. In fact, salinity provoked an increase of Mn-SOD activity by 60% in in the WT and KO-2 plants and by 190% in the KO-1 genotype. Fe-SOD was not modified in the WT while in *trxo1* mutant plants increased (more than double in KO-1 and by 20% in KO-2). Moreover, Cu, Zn-SOD increased more in the mutant plants (by 85%) than in the WT (by 30%).

Catalase activity, a H_2O_2 scavenging enzyme, was higher in the *trxo1* mutants than in WT plants under both control and saline conditions (Fig. 7C). This activity decreased similarly (by 50%) in all genotypes under salinity. Another key enzyme involved in the scavenging of H_2O_2 is APX, an ascorbate specific peroxidase of the ASC-GSH cycle whose activity was not modified by the presence or absence of TRX*o*1 (Fig. 8A), neither under control nor under saline conditions. This activity increased of about 2-fold in all plants exposed to the salt treatment.

The enzymatic regeneration of the oxidized forms of ASC is controlled by two enzymes of the ASC-GSH cycle, MDHAR and DHAR. MDHAR was similar in all genotypes under control conditions (Fig. 8B) and it decreased 3-fold as a result of salinity. Upon salt treatement, KO-1 plants showed a lower MDHAR activity than WT and KO-2 plants. DHAR had a higher activity in the KO-2 mutant under control conditions, while DHAR acrivity was lower in KO-2 under salinity compared to that in WT and KO-1 genotypes (Fig. 8C). GR activity, the last component of the ASC-GSH cycle involved in the regeneration of GSH from GSSG was similar in all genotypes under control conditions (Fig. 8D). Salinity strongly induced this activity (between 6-9-fold) in all genotypes with the highest increased observed for the KO-1 plants.

Discussion

KO *trxo1* mutants do not present an evident phenotype

The lack of appreciable phenotype in the *trxo1* mutant plants in control or even under saline conditions (Fig. 1) suggests that the AtTRX*o*1 protein function may be compensated. In fact, most thioredoxin mutants do not present obvious phenotype and it could be due to redundancy among TRXs as it is a multigenic family or with other proteins displaying similar function as TRXs (Brehelin et al. 2004). Recently Daloso et al. (2015) have described a lack of phenotype in 8-week old plants of the same *trxo1* mutant (line 374, KO-2), similar to that found in the present work, although the 4-week old mutants had a lower fresh weight than the WT genotype. The different behavior could be due to the conditions of growth in both studies. A lack of phenotype has been also observed for KO mutants of cytosolic/mitochondrial NTRA and NTRB (Reichheld et al. 2007) as well as for the mitochondrial PRXIIF (Finkemeier et al. 2005). However, the differences in stomata density and aperture of the two *trxo*1 mutant lines that we observed (Fig. 4B) could have possible consequences for the plants metabolism as discussed below.

Salinity stress affects differently between WT and KO *trxo1* plants

Physiological parameters normally reflect plant response or tolerance to stress environments. Plants require macronutrients in relatively large amounts (>0.1% of dry mass) to complete their life cycle. The accumulation of Na at high concentrations in NaCl-treated plants, accompanied by a modest reduction in K accumulation, is a characteristic response in many plants (Zhu 2001), and the lower accumulation of Na found in the *trxo1* mutants (Table 1) implies a different regulation than in WT plants, either due to a less efficient accumulation or a more efficient exclusion. In Brassicaceae, the tolerance of the tissues to accumulated Na seems to be more important than the process of exclusion (Moller and Tester 2007) with some exceptions in the family. Magnesium is a constituent of the chlorophyll molecule so its deficiency leads to pale-green to yellow plant leaves with interveinal chlorosis (Hermans and Verbruggen 2008), although this symptom was not observed in our trx*o1* mutants, in spite of their lower Mg content compared to the WT in control conditions. On the other hand, manganese is not a constituent of chlorophyll, but it is vital for the functionality of the PSII in photosynthesis (Sun et al. 2001) and its deficiency is another cause of leaf chlorosis (Eroglu et al. 2016). The observed increase in this micronutrient in the mutants grown under salt conditions could contribute to their adequate response to the imposed stress conditions. Salinity provokes a reduction in the photosynthetic process in different species with C3 metabolism including pea and Arabidopsis (Chaves et al. 2009, Martí et al. 2011, Stepien et al. 2009). Also related, high concentrations of Na are required to inhibit some enzymes involved in photosynthesis (Munns et al. 2006) and enzymes that require K are particularly sensitive to high Na concentration or high Na/K ratio (Chaves et al. 2009). The fact that our KO-2 mutant has a lower Na content could be affecting the photosynthetic response which, on the other hand, is also influenced by the gas exchange through stomata. In fact, enhanced stomatal conductance through either increased stomatal density or opening may be detrimental and result in a reduced survival rate under unfavorable conditions (Flexas et al. 2014). The decreased and

similar g_s presented by KO mutants compared to WT plants (Fig. 3A) may reflect an adaptative response under the saline conditions imposed. Stomatal pores regulate water evaporation, leaf temperature and gas exchange including uptake of CO_2 to provide with the carbon source for photosynthetic reactions. An excess water loss under drought or salinity might be dangerous resulting in plant death, so a proper stomata movement is essential for adaptation. Several signaling systems control the aperture and closure of stomata including exogenous stimuli as light or stress and endogenous as phytohormones, ABA being the main one involved in the closure, together with H_2O_2, NO, and Ca ions (Kollist et al. 2014, Murata et al. 2015). Variation in the rate of water loss on excised plants or leaves is mainly affected by constitutive differences in initial stomatal conductance (prior to stomatal closure), the sensitivity of stomata to water deficit and in lesser extent by cuticular conductance (Juenger et al. 2005, Kerstien et al. 2006). In our plants, initial stomatal conductance was similar in all genotypes and the water loss phenotype observed in the fully developed leaves of KO *trxo1* mutants grown under normal environment conditions seems not caused by physiological differences in stomata (Fig. 3), although their higher stomatal density together with the lower stomatal aperture found in these leaves, could explain the observed differences in water retention in relation to WT (Fig. 4). Under salinity, the KO mutants, mainly KO-1, retain more water thanks to a higher stomatal closure, which may be an important factor collaborating to an adequate response to salinity, probably influencing CO_2 assimilation. In this way, TRX*o*1 seems to be involved in stomatal aperture under both normal and salinity conditions, and further study on the photosynthetic metabolism is being actually carried out to understand the possible involvement of TRX*o*1 in this process.

NO acts as a signaling molecule with multiple biological functions in plants such as the induction of seed germination, the regulation of plant maturation, senescence or response to biotic and abiotic stress including salinity (Bellin et al. 2013, Camejo et al. 2013, Martí et al. 2013, Rodríguez-Serrano et al. 2006), and is also involved in stomatal closure. The fact that *trxo1* mutants had a higher NO content than WT plants under control conditions (Fig. 5A) could affect their response to salinity which provoked a slight increase in this molecule, probably indicating that the level of stress is not very severe and that the mutant plants have an effective mechanism to maintain their NO content. The similar H_2O_2 level found in WT and *trxo1* plants grown under control conditions (Fig. 5B), together with the maintained level of lipid peroxidation in the mutants (Fig. 5C) imply a lack of an important oxidative damage of membranes due to the absence of TRX*o*1 in the plants, although the oxidation of proteins measured as carbonyl groups is affected. The observed increase in H_2O_2 content, lipid peroxidation and protein oxidation levels in all the genotypes as a consequence of salinity has been widely reported. As for other plants, ROS production is increased under salt stress in the different cell compartments leading to oxidative stress but also as a part of the signaling pathway involved in the plant response (Hernández et al. 1993, 2000, 2001, Xie et al. 2011). Specifically, salinity is known to provoke an oxidative stress through the ROS production, and the antioxidant system, including the

mitochondrial one that presents a high plasticity and usually responds to adverse environments (Lázaro et al. 2013). Generally, the level of lipid peroxidation as an indicator of oxidative damage is lower in salt tolerant plants than in sensitive plants exposed to a salt stress condition. In summary, the level of NaCl applied to Arabidopsis in our study seems to provoke an oxidative stress in terms of membrane damage, mainly in the *trxo1* plants. The mutants also presented higher H_2O_2 and NO contents, which may influence the closure of stomata. All these results indicate that different defence mechanisms are operating in the response of the genotypes to salinity, pointing TRX*o*1 as involved in the adaptation.

The antioxidant system responds to the lack of AtTRX*o*1 under salinity

Together with their above-mentioned deleterious effects, ROS act as signaling molecules allowing plant acclimation to stress through the induction of antioxidant defenses, including the components of the ascorbate-glutathione cycle and the enzymes SOD, catalase and peroxidase (POX) (Camejo et al. 2013, Foyer and Noctor 2013, Hernández et al. 1999, 2000). However, the information related to the role of mitochondrial TRX*o*1 in plants under stress is scarce. In silico studies of Arabidopsis *TRXs* has revealed that *AtTRXo1* gene expression does not vary as a response to several stress situations including salinity (Belin et al. 2015). However, in pea plants, PsTRX*o*1 is a component of the antioxidant mitochondrial response providing the cell with a defense mechanism against NaCl (Martí et al. 2011). An adaptative response exists against short treatments with 150 mM NaCl in pea plants increasing gene expression while longer treatment provoked a down regulation of the gene in parallel to an increase in PsTRX*o*1 protein and TRX oxidoreductase activity. The induction of the activity was correlated with the increase in the capacity of AOX, as well as a higher demand to regenerate the oxidized form of PRXIIF (Martí et al. 2011), probably influencing the higher levels of H_2O_2 found in our KO mutants under salinity. Furthermore, higher amount of TRX*o*1 in *PsTRXo1* over-expressing tobacco BY-2 cells protected them against induced oxidative stress by exogenous H_2O_2 treatment, increasing some antioxidant enzyme activities and their viability (Ortiz-Espín et al. 2015), pointing TRX*o*1 as a key component in the defense against oxidative stress.

In this work, the absence of AtTRX*o*1 in plants grown under control conditions provoked a change in the activities of SOD and catalase, enzymes involved in the H_2O_2 metabolism (Fig. 7). SOD is a crucial enzyme that eliminates ROS in plant cells, its role under environmental stresses is widely studied (Alscher et al. 2002, Gómez et al. 1999, Sandalio et al. 2001). Mn-SOD isoenzyme controls $O_2^{\cdot-}$ content in the mitochondria and allows the organelle to regulate the internal H_2O_2 concentration and salt stress response (Hernández et al. 1993, Sevilla et al. 1982). Usually, an increased antioxidant activity can prevent the oxidative damage accompanying salt stress. As an example, a correlation was found between Mn-SOD expression, protein and activity levels and the tolerance to salt in resistant cultivars in pea, wheat or tomato, which showed higher activities of this isoenzyme than sensitive cultivars (Hernández et al. 1993, Martí et al. 2013, Mittova et al. 2003, Sairam and Srivastava 2002).

Moreover, overexpression of Mn-SOD in Arabidopsis was accompanied by an increased salt tolerance and also by a higher increase in the activities of Cu/Zn-SOD, Fe-SOD, POX and CAT compared to what observed in WT plants (Wang et al. 2004). The lack of mitochondrial TRX*o*1 in the Arabidopsis mutant plants grown under salinity provoked a high induction of SOD isoenzymes, not only Mn-SOD but also Fe- and Cu/Zn-SOD isoenzymes that could be compensating the TRX*o*1 deficiency and maintaining higher levels of H_2O_2 than in the WT plants, probably also important for signaling. In this condition, the observed increase in Zn and Mn in the *trxo1* mutants could be necessary to maintain the enzymatic activities. Under our saline conditions, the lower catalase activity together with the increase in SOD may be in part responsible for the increased H_2O_2 observed in WT and *trxo1* plants (Fig. 7). However, *trxo1* plants presented higher catalase activity under control conditions but also under salinity, although the differences were lower in the stress conditions.

The control of H_2O_2 is also exerted by the ASC-GSH cycle components which play an important role in stress situations. GSH is an essential thiol antioxidant acting as a general redox buffer as a ROS scavenger and it also serves as a mobile carrier of reduced sulfur (Schnaubelt et al. 2015). In our work, the lack of TRX*o*1 did not result in an accumulation of reduced or oxidized forms of glutathione under control conditions (Fig. 6). Similarly, inactivation of NTR in Arabidopsis mutants did not produce GSSG accumulation, synthesis of GSH or any modification in the redox state of this antioxidant (Reichheld et al. 2007). However, in yeast *trx1/trx2* double mutants, an increase in glutathione content (mainly GSSG) has been reported (Muller 1996). This putative compensation has been also observed when the gene expression of two *TRX h* was shown to increase in GSH-deficient mutants (Schnaubelt et al. 2015) or in Tobacco BY-2 cells over-expressing *PsTRXo*1 presenting lower levels of GSH than non over-expressing cells (Calderón et al. 2017, Ortiz-Espín et al. 2015). From a physiological point of view, the glutathione redox state could be more influential in explaining the effects of different stresses, in the control of gene expression and protein function (Gómez et al. 2004, Rellán-Álvarez et al. 2006, Schnaubelt et al. 2015). Under salinity and drought conditions, a high glutathione redox state plays an important role in plants such as pea, tomato or wheat (Hernández et al. 2000, Kocsy et al. 2002, Shalata et al. 2001). Thus, the GSH recycling, including its reduction from oxidized state, is a key factor determinant in the tolerance to this kind of abiotic stresses. Specifically under salinity, increases in the GR activity have been positively correlated to tolerance (Gómez et al. 1999, Lázaro et al. 2013). Under our saline conditions, GR activity was highly increased in all genotypes and may contribute to the observed maintenance in the reduced form GSH. Moreover, the imposed stress does not seem to be severe enough to induce an important change in the redox state, and thus, GR activity may contribute to the existence of an efficient response of the plants under these unfavorable conditions.

Ascorbate is also an important plant antioxidant with a significant role in growth, development, and stress responses (Wang et al. 2013). In our work, the observed increase in ASC in WT plants grown under salinity was not present in the *trxo1* mutants (Fig. 6D). The lower ASC content in the KO-1

mutant was accompanied by a lower MDHAR activity (Fig. 6B) involved in its regeneration from the oxidized form. It has been described that a lower ascorbate content in the Arabidopsis *vitc* mutant provoked an increased sensitivity to NaCl stress, causing an increased oxidative stress mainly due to an impairment of ASC-GSH cycle (decreased DHAR and MDHAR activities) and photosystem II function (Huang et al. 2005). On the other hand, the KO-2 mutant had a decreased DHAR activity under salinity that was not accompanied by a significant decreased in ASC content and thus, the synthesis of this antioxidant may be important for the maintained levels observed in these plants. Interestingly, the unexpected differences between the two *trxo1* mutant lines under salinity, mainly in the response of the ASC-GSH cycle enzymes, is similar to that described for KO mutants of the target protein AtPRXIIF, with one line being less affected by cadmium (inhibition of root growth) than the other possibly due to higher activities of APX and GPX (Finkemeier et al. 2005). In several species including pea plants, salt tolerance is correlated with increase in the activity of the antioxidant system or with higher endogenous levels (Lázaro et al. 2013). In this way, the response of the antioxidant system would contribute to guarantee the adaptive response and physiological activity not only in WT plants but also in *trxo1* mutants in which mainly catalase and the different SOD isozymes and GR may be important in the adaptative response.

Recently, Daloso et al. (2015) have shown the importance of the TRX system in mediating the metabolic control of the mitochondrial tricarboxylic acid cycle, pointing to the redox mechanism as a broadly operative system for regulating this cycle. Also, the lack of TRX*o*1 is likely affecting fundamental processes in mitochondria in which TRX*o*1 is involved such as photorespiration. In fact, a decrease in serine content was observed in the *trxo1* mutant possibly due to the lack of regulation of TRX-linked mitochondrial enzymes of the photorespiratory pathway (Daloso et al. 2015). One of these enzymes maybe serine hydroxymethyl transferase, a mitochondrial key enzyme in the photorespiration process and a protein described as a possible target of TRX*o*1 in mitochondria from pea plants (Martí et al. 2009). Subcellular studies in mitochondria are being carried out to try to deepen the role of TRX*o*1 in situations where processes like mitochondrial respiration or photorespiration are fundamental for the response of plants, such as the growth of plants under salinity conditions.

Conclusions

In summary, the transgenic Arabidopsis plants lacking TRX*o*1 in mitochondria respond well to 100 mM NaCl, similarly to that found for WT plants. It suggests that internal cell mechanisms must be operating in order to compensate the lack of the protein for protecting cell integrity under these stressful conditions. This study has shown that the activities of antioxidative enzymes such as Mn-SOD, Fe-SOD, Cu/Zn-SOD, GR and catalase in transgenic plants under salt stress were higher than those of WT, indicating a compensating response, although higher H_2O_2 content and lipid peroxidation

were found in these plants. H_2O_2 could act as signaling molecule in this adverse situation, maybe allowing stomatal movements to avoid higher loss of water, among others. Understanding the role of TRX*o*1 through its action on specific target proteins and the mechanisms of adaptive responses of plants under environmental stresses in the different cell compartments, will provide useful information for the agronomic use of degraded areas as consequence of salinity in the soil or in the irrigation water.

Author contributions

A.J. and F.S. designed the research. A.C., A.S-G., I.M., A.O. and D.C. performed the experiments. A.C., A.O. and A.S-G. analysed the data and A.J. and F.S. wrote the paper.

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Figure legends

Fig 1. (A) Representative 21-day old Arabidopsis wild type (WT) and two KO *AtTRXo1* mutant lines growing during 7 days in the absence (control) or presence of 100 mM NaCl, (B) rosette diameter, (C) leaf width and (D) number of leaves of WT and *AtTRXo1* mutant plants grown in the absence (control) and presence of 100 mM NaCl. Data are the mean ± SE of at least four different experiments. The different letters indicate significant differences ($P<0.05$) among genotypes in each condition according to the Tukey´s test, and asterisks indicate significant differences of each genotype under salinity compared with control condition using the *t*-Student test ($P<0.05$).

Fig 2. (A) Fresh weight and (B) dry weight of WT and two KO *AtTRXo1* mutant plants grown in the absence (control) and presence of 100 mM NaCl. The different letters indicate significant differences (P<0.05) among genotypes in each condition according to the Tukey´s test, and asterisks indicate significant differences of each genotype under salinity compared with control condition using the *t*-Student test (P<0.05).

Fig 3. (A) Stomatal conductance (g_s) and water loss in (B) control and saline (C) conditions of WT and mutant plants in which the fresh weight of the rosettes was measured every 10 min during 2 h. Data are the mean ± SE of at least four different experiments. The different letters indicate significant differences (P<0.05) among genotypes in each condition according to the Tukey´s test, and asterisks indicate significant differences of each genotype under salinity compared with control condition using the *t*-Student test (P<0.05).

Fig 4. (A) Stomata visualization (representative picture) of WT and two KO *AtTRXo1* mutant plants grown in the absence (control) and presence of 100 mM NaCl. (B) Stomatal density and (C) aperture index calculated by division of the stomatal aperture width through the length. Data are the mean ± SE of at least four different experiments. The different letters indicate significant differences (P<0.05) among genotypes in each condition according to the Tukey´s test, and asterisks indicate significant differences of each genotype under salinity compared with control condition using the *t*-Student test (P<0.05).

Fig 5. (A) Nitric oxide, (B) hydrogen peroxide, (C) malondialdehyde (MDA) and (D) carbonylated (CO) proteins in WT and two KO *AtTRXo1* mutant plants grown in the absence (control) and presence of 100 mM NaCl. Data are the mean ± SE of at least three different experiments. The different letters indicate significant differences (P<0.05) among genotypes in each condition according to the Tukey´s test, and asterisks indicate significant differences of each genotype under salinity compared with control condition using the *t*-Student test (P<0.05).

Fig 6. (A) Reduced (GSH) and (B) oxidized (GSSG) glutathione, (C) nitrosoglutathione (GSNO) and (D) ascorbate (ASC) content in WT and two KO *AtTRXo1* mutant plants grown in the absence (control) and presence of 100 mM NaCl. (E) Table presenting the redox state of glutathione as percentage of GSH/total glutathione. Data are the mean ± SE of at least three different experiments. The different letters indicate significant differences (P<0.05) among genotypes in each condition according to the Tukey´s test, and asterisks indicate significant differences of each genotype under salinity compared with control condition using the *t*-Student test (P<0.05).

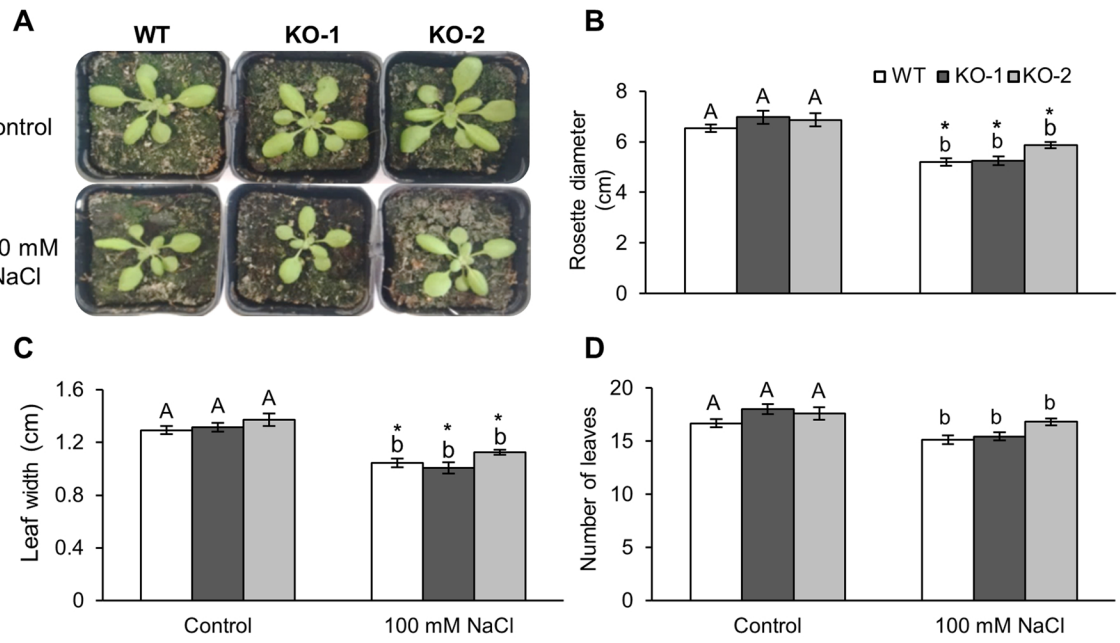
Fig 7. (A) Total and (B) isoenzyme activity (after PAGE) of superoxide dismutase (SOD) and (C) catalase activity in WT and two KO *AtTRXo1* mutant plants grown in the absence (control) and presence of 100 mM NaCl. The table presents the specific activity of the different SOD isoenzymes

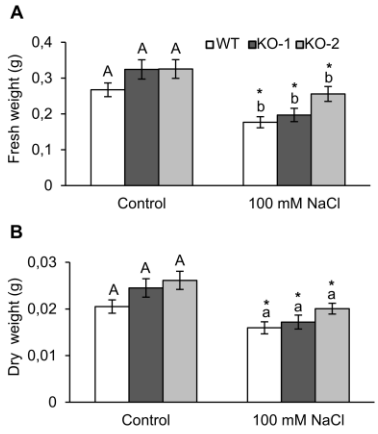
after PAGE analysis. Data are the mean ± SE of at least three different experiments. The different letters indicate significant differences (P<0.05) among genotypes in each condition according to the Tukey´s test, and asterisks indicate significant differences of each genotype under salinity compared with control condition using the *t*-Student test (P<0.05).

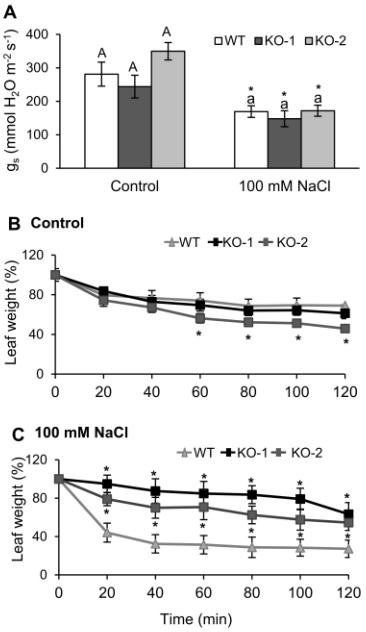
Fig 8. Ascorbate-glutathione cycle enzymatic activities in WT and two KO *AtTRXo1* mutant plants grown in the absence (control) and presence of 100 mM NaCl. (A) Ascorbate peroxidase (APX), (B) monodehydroascorbate reductase (MDHA), (C) dehydroascorbate reductase (DHA) and (D) glutathione reductase (GR). Data are the mean ± SE of at least three different experiments. The different letters indicate significant differences (P<0.05) among genotypes in each condition according to the Tukey´s test, and asterisks indicate significant differences of each genotype under salinity compared with control condition using the *t*-Student test (P<0.05).

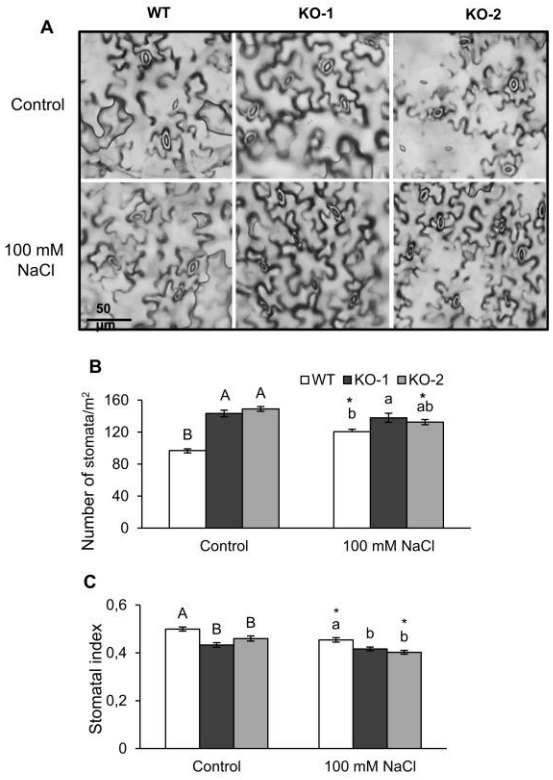
Table 1. Nutrient content in leaves of WT and two KO *AtTrxo1* mutant plants grown in the absence (C) and presence (treated, T) of 100 mM NaCl. Data are the mean ± SE of at least three different experiments. The different letters indicate significant differences (p < 0.05) according to the Tukey´s test.

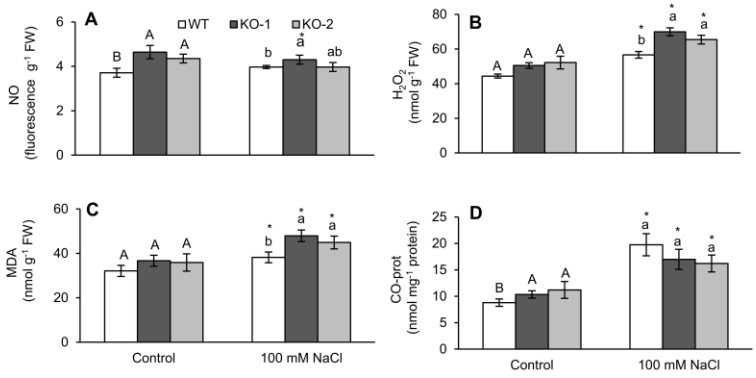
	Ca	**Na**	**K**	**P**	**Mg**	**S**	**Zn**	**Mn**
	g 100 g^{-1} dry weight						mg kg^{-1} dry weight	
WT-C	3.45±0.12^{a}	0.21±0.01^{c}	4.46±0.12^{a}	0.92±0.01^{a}	0.42±0.02^{a}	0.99±0.01^{a}	122±4^{b}	265±8^{ab}
KO1-C	3.11±0.18^{b}	0.19±0.01^{c}	4.71±0.10^{a}	0.84±0.01^{b}	0.36±0.01^{b}	0.96±0.02^{a}	126±1^{b}	246±9^{b}
KO2-C	3.56±0.12^{a}	0.08±0.01^{c}	4.45±0.10^{a}	0.64±0.06^{c}	0.38±0.01^{b}	0.60±0.01^{c}	85±4^{d}	254±23^{b}
WT-T	3.50±0.10^{a}	0.99±0.04^{a}	3.08±0.09^{b}	0.88±0.01^{a}	0.42±0.01^{a}	0.87±0.01^{b}	121±4^{b}	247±9^{b}
KO1-T	3.29±0.06^{b}	0.79±0.05^{b}	3.32±0.08^{b}	0.84±0.02^{b}	0.42±0.01^{a}	0.90±0.01^{b}	171±8^{a}	286±10^{a}
KO2-T	3.68±0.08^{a}	0.24±0.13^{c}	3.18±0.11^{b}	0.65±0.02^{c}	0.42±0.01^{a}	0.65±0.03^{c}	101±6^{c}	266±11^{a}

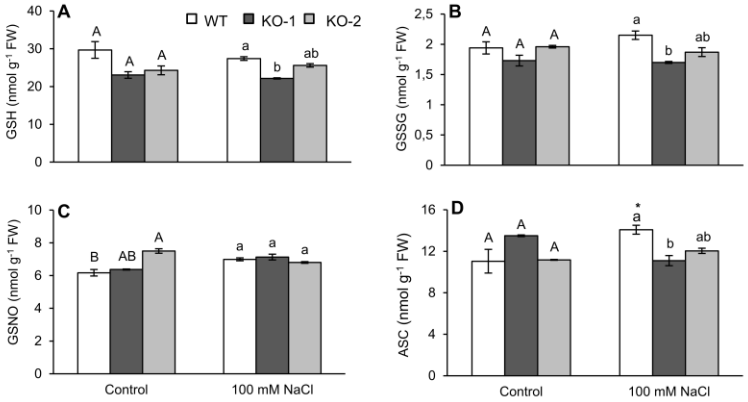








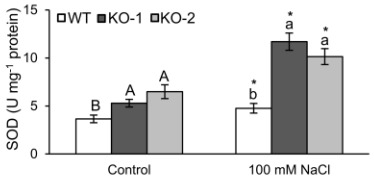




E

	GSH/GSH+GSSG (%)		
	WT	**KO-1**	**KO-2**
Control	93.8±0.1	93.0±0.1	92.4±0.2
100 mM NaCl	93.0±0.1	92.9±0.05	93.2±0.1

A



B

	Mn-SOD	Fe-SOD	Cu/Zn-SOD
	U mg^{-1} protein		
WT-C	0.65	1.17	1.83
KO-1-C	1.05	1.93	2.31
KO-2-C	1.33	2.24	2.92
WT-T	1.04	1.30	2.42
KO-1-T	3.07	4.26	4.37
KO-2-T	2.04	2.70	5.40

C