Coordinated responses of mitochondrial antioxidative enzymes, respiratory pathways and metabolism in *Arabidopsis thaliana* thioredoxin *trxo1* mutants under salinity

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Running title: Mitochondrial metabolism in *trxo1* mutants under salinity

Abbreviations. Alternative oxidase (AOX), ascorbate (ASC), ascorbate peroxidase (APX), catalase (CAT), cytochrome c pathway (COX), dehydroascorbate reductase (DHAR), ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), glutathione (GSH), glutathione reductase (GR), knock-out (KO), monodehydroascorbate reductase (MDHAR), N-ethyl-maleimide (NEM), peroxiredoxin (Prx), phenylmethanesulfonyl fluoride (PMSF), superoxide dismutase (SOD), thioredoxin (Trx), tricarboxylic acid (TCA), wild type (WT)

Abstract

Plant cells suffer alterations of their redox state and increase mitochondrial ROS generation during salinity. To avoid this, they activate several mitochondrial antioxidant and redox systems including the alternative oxidase (AOX), superoxide dismutase (SOD) and the ascorbate-glutathione (ASC-GSH) cycle components in a coordinated manner. The redox-sensitive mitochondrial thioredoxin (Trx) system may be responsible for this coordination through the redox regulation of target proteins. On top of this, metabolic perturbations induced by salinity may lead to alterations of the redox state of the Trx system. In order to explore the association between redox and metabolic changes occurring in mitochondria under saline conditions, we analyzed the salt-stress responses of mitochondrial antioxidant systems and metabolism in wild type (WT) and two knock-out (KO) *AtTrxo1* lines. The activities of Mn-SOD and components of the

ASC-GSH cycle were determined in isolated mitochondria, together with an evaluation of the AOX redox state, the oxidative stress, and catalase activity. Moreover, the *in vivo* activities of cytochrome (COX) and alternative mitochondrial respiratory pathways and primary metabolites profile were determined. Our results show that the lack of Trx*o*1 neither resulted in oxidative stress at the mitochondrial level nor in an upregulation of the antioxidant enzymes under salinity, although glutathione reductase (GR) maintained its high constitutive level as observed in control conditions. Moreover, the AOX was found invariably in its reduced monomeric state and displayed a reduction of its *in vivo* activity in all genotypes after the salt treatment, probably due to the mild severity of the treatment. Interestingly, *trxo1* mutants displayed altered patterns in AOX isoforms and in the activities of the ASC-GSH cycle components and the electron partitioning to the AOX pathway indicating a reorganization of the different antioxidant systems. Furthermore, decreases on glucose and fructose levels in both *trxo1* mutants coincided with an increased respiration through the COX pathway under control conditions. All these changes collaborate to maintain a low oxidative stress and the energy demand in both, control and salinity conditions and reflect the acclimation of all the genotypes to the applied stress.

Keywords. Alternative oxidase**,** ascorbate-glutathione cycle, reactive oxygen species (ROS), respiration, salinity, thioredoxin *o* mutants

1. Introduction

Mitochondria are key organelles involved in ROS generation during plant development and also play an essential function under stress situations such as salinity where ROS production is increased (Hernández et al., 1993; Gómez et al,. 1999; Sevilla et al., 2015a; Huang et al., 2016). Salinity is one of the main environmental constraints which limit plant productivity in spite of the induction of defence systems, affecting several cellular processes with an associated oxidative and nitrosative stress (Gómez et al., 2004; Camejo et al., 2013). Among others, salt stress affects respiration, with the impact depending on the sensitivity of the plant species and the duration and severity of the imposed stress (Koyro et al., 2006; Jacoby et al., 2011; Martí et al., 2011; Del-Saz et al., 2016). Several antioxidant and redox systems participate in the protection of

mitochondria from the oxidative stress induced by salinity. In seed plants, the mitochondrial electron transport chain (mETC) presents a phosphorylating cyanide-sensitive cytochrome c oxidase (COX) pathway in parallel to a non-phosphorylating cyanide insensitive alternative oxidase (AOX) pathway that directly couples the oxidation of ubiquinol with the reduction of oxygen to water. A general role of the AOX pathway is related to the avoidance of over-reduction of the ubiquinone pool which reduces the possibility of ROS production (Del-Saz et al., 2018). Moreover, it can allow the continuation of the tricarboxylic acid (TCA) cycle when there is a limitation on the NAD(P)H re-oxidation (Del-Saz et al., 2018). Salinity can promote both high ROS production and a decrease on the energy demand for growth and therefore the AOX has been proposed to play an important role on the response of plant metabolism to salt stress (Smith et al., 2009; Martí et al., 2011; Del-Saz et al., 2016). Transcript levels of AOX genes are highly responsive to salt stress and plants overexpressing *AtAOX1a* present lower ROS formation and improved growth under saline conditions (Smith et al., 2009). At the protein level, the AOX is post-translationally regulated by two mechanisms that involve a redox regulation yielding reduced active dimers which can subsequently be further activated by its interaction with different organic acids (Selinski et al., 2018). The candidate proposed to redox regulate the AOX is the mitochondrial thioredoxin which has indeed been shown to activate the AOX *in vitro* (Martí et al., 2009; Del-Saz et al., 2018). In fact, the level of reduction and the protein amount were suggested to be essential in determining the extent of the AOX activity *in vivo* (Ribas-Carbó et al., 1997; Martí et al., 2011) although this is still a matter of debate (Del-Saz et al., 2018). The *in vivo* AOX and COX activities can only be determined by using the oxygen isotope fractionation technique and such studies under saline conditions are still scarce (Del-Saz et al., 2018). Another key antioxidant enzyme in the mitochondria is superoxide dismutase (SOD) which is responsible for eliminating $O_2^{\cdot-}$ to generate H_2O_2, and which respond to salinity depending on the sensitivity of different genotypes (Olmos et al., 1994; Ashraf et al., 2009; Hafsi et al., 2010). The coordinated action of Mn-SOD and AOX proteins in response to stress is also thought to reduce the $O_2^{\cdot-}$ levels thus diminishing the harmful effects of oxidative stress (Martí et al., 2011; Sevilla et al., 2015b). In collaboration with these systems, the ascorbate-glutathione (ASC-GSH) cycle components are coordinated to eliminate the H_2O_2 generated (Jiménez *et al*., 1997). The enzymes of this cycle display a high plasticity in their gene regulation and are important components in the

maintenance of an adequate redox state of the antioxidants ASC and GSH under stress conditions (Lázaro et al., 2013; Ortiz-Espín et al., 2017a; Hossain et al., 2018). Furthermore, the reduced/oxidized balance of these two antioxidants is crucial for the cell stress signaling responses (Foyer and Noctor, 2011) due to their influence on stress-responsive gene expression (Munné-Bosch et al., 2013).

In addition to the above-mentioned antioxidant systems, thiorredoxins (Trx) peroxiredoxins (Prx), sulfiredoxins (Srx) and glutaredoxins (Grx) are redox-sensitive proteins present in mitochondria which may be involved in the regulation of the plants response to salinity (Rouhier et al., 2006; Barranco-Medina et al., 2007; Guo et al., 2010; Iglesias-Baena et al., 2011; Martí et al., 2011; Sevilla et al., 2015a). The mitochondrial Trx isoforms belong to the *h* or *o* classes (Laloi et al., 2001; Gelhaye et al., 2004; Martí et al., 2009). The Trx*o*1 protein has been described in Arabidopsis mitochondria while in pea nucleus and mitochondria, where it may regulate the redox state of different target proteins (Martí et al., 2009; Calderón et al., 2017), including mitochondrial AOX and peroxiredoxin PrxIIF, exerting a signaling function during salt stress (Barranco-Medina et al., 2008, 2009; Martí et al., 2009). Interestingly, post-translational modifications play a key role in regulation of these proteins under stress conditions including salinity (Horling et al., 2003; Finkemeier et al., 2005; Martí et al., 2011). Pea PrxIIF was found as *S*-nitrosylated under long-term saline conditions (Camejo et al., 2013) and this modification has been described to provoke a conformational change and functional switch in the protein from peroxidase to transnitrosylase, which could probably function as a protective mechanism under conditions inducing oxidative and nitrosative stress (Camejo et al., 2013, 2015). Recently, it has been reported that recombinant mitochondrial AtTrx*o* showed capacity to bind an Fe-S cluster, although the physiological relevance of this observation remains unclear (Zannini et al., 2018).

In order to gain more insight into the physiological function of Trx*o*1, we have analysed the salt-stress responses of mitochondrial antioxidant systems and metabolism in wild type (WT) and two Knock out (KO) *AtTrxo1* (named *trxo1*) lines. Recently, an increase in antioxidant enzymes SOD, GR or catalase has been reported at the leaf level in Arabidopsis KO *trxo1* mutants under salinity, probably to compensate the lack of Trx*o*1 in mitochondria (Calderón et al., 2018) and a contribution of the mitochondrial Trx system under drought stress in Arabidopsis has been reported (da Fonseca-Pereira

et al., 2018). However, the impact of the lack of Trx*o*1 in the antioxidant system in isolated mitochondria is unknown. In this respect, the activities of Mn-SOD and components of the ASC-GSH cycle were determined in the organelles together with an evaluation of the AOX redox state and the oxidative stress possibly imposed by the saline condition. The study of the AOX pathway *in vivo* in the *Trxo1* mutants under salinity is two-fold relevant. Firstly, different roles of the AOX pathway have been proposed in the tolerance of plants to salinity stress (Smith et al., 2009; Martí et al., 2011; Del-Saz et al., 2016; Hossain and Dietz, 2016) but its complex interplay with other antioxidant systems, including Trx*o*1, remains poorly explored. Secondly, Trx*o*1 has been proposed as the physiological candidate activating the AOX in pea, poplar and Arabidopsis plants (Martí et al., 2009; Yoshida et al., 2013), particularly under saline conditions (Martí et al., 2011). In this paper, the *in vivo* activities of COX and AOX mitochondrial respiratory pathways and primary metabolites profile have been determined in order to explore the links between redox and metabolic changes caused by the absence of *AtTrxo1* under saline conditions.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of *Arabidopsis thaliana* L. wild-type (ecotype Columbia) and two T-DNA insertion (At2g35010) KO-*AtTrxo1* lines in this ecotype (SALK_143294C, line 590 (named KO1) and SALK_042792 line 374 (named KO2) were obtained from the European Arabidopsis Stock Centre (NASC; http://arabidopsis.info/; Nottingham University, UK). The homozygous plants were selected as described in Ortiz-Espín *et al*. (2017b) where the lack of *AtTrxo1* expression was shown in KO1 while a residual expression was observed in KO2 plants.

Plants (around 120 seeds per plate) were grown as described by Wallström et al. (2014) with some modifications. Seeds were sterilized and sown on plates prepared with 2.5 g/L of MS medium, 18 g/L sucrose (pH 5.7) supplied with 8 g/L plant agar. Sown seeds were stratified at 4ºC for 3 days and then plates were maintained under controlled conditions of light (80 µmol/m^2/s photosynthetic active radiation), photopheriod (16 h

light/8 h dark), relative humidity (60%) and temperature (22/18ºC light/dark) for 21 days. For the salt treatment, 100 mM NaCl was added to the plant growth medium.

2.2. Growth analysis

Growth of WT and KO lines in the absence and presence of 100 mM NaCl was evaluated by measuring fresh weight, rosette diameter, number of leaves and root length in 21-old plants.

2.3. Isolation of mitochondria

Mitochondria were isolated by differential and density Percoll gradient centrifugation using 10-15 g of rosette leaves of the Arabidopsis plants as described by Keech et al. (2005) with the following modifications: a grinding medium: 0.25 M sucrose, 1.5 mM EDTA, 4 mM cysteine, 15 mM MOPs-KOH (3.14 g/L), pH 7.4, 0.4 % BSA, 0.6 % PVP-40; a washing buffer: 0.3 M sucrose, 1 mM EGTA, 0.2 mM PMSF, 10 mM MOPs-KOH pH 7.2 and a 27/45% Percoll gradient in a buffer containing: 0.3 M sucrose, 10 mM MOPs-KOH, pH 7.2. For APX extraction, 20 mM ascorbic acid was included in the grinding buffer and 2 mM in the other buffers, and EDTA/EGTA were omitted. For SOD activity and AOX protein content, an enriched-mitochondrial extract (pellet from 18000*g* resuspended in washing buffer) was used, and in case of AOX, all buffers contained 50 mM NEM.

2.4. Western blotting

Total seedling extracts were prepared from 0.5 g of frozen tissue ground to a powder with liquid nitrogen using 2 ml SDS-PAGE Laemli (1970) sample buffer without reductant. On the other hand, enriched-mitochondrial extracts were obtained as described in the previous section, treated with triton 0-.1% X-100 and incubated 30 min at 4ºC. All samples were centrifuged at 14,000g for 10 min and supernatant were boiled for 5 min and proteins were separated by SDS-PAGE according to the method of Laemmli (1970). Subsequently proteins were electrotransferred to nitrocellulose filters

using a blot-transfer buffer (48 mM Tris, 134 mM Gly, 0.03% SDS, 20% [v/v] methanol). 60 μg of proteins from total extracts and 25 μg of mitochondrial-enriched extracts were used in each lane. Monoclonal mouse AOX antibody (1:50) (Elthon et al., 1989) and goat anti-mouse antibody IgG-HRP: sc-2031 (Santa Cruz Biotechnology Inc.) were used as primary and secondary antibodies, respectively. AOX protein was detected using ECL Plus Western blotting detection system (GE Healthcare, Hertfordshire U.K.). To quantify the bands we applied Quantity One software-based analysis (BioRad).

2.5. Lipid peroxidation and carbonyl proteins

The extent of lipid peroxidation was estimated by determining the concentration of substances reacting with thiobarbituric acid as described in Ortiz-Espín et al. (2015). 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 95 ^{o}C 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 using 2,4-dinitrophenylhydrazine (DNPH) as described by Ortiz-Espín et al. (2015). The total soluble protein content was quantified using the Bradford method (Bradford, 1976).

2.6. Enzyme assays

Mitochondrial extracts were used for enzymatic determinations, being previously treated with 50 mM phosphate buffer pH 7.8 containing 0.1% (v/v) Triton X-100 during 5 min at 4ºC.

The yield of purified mitochondria was estimated from the cytochrome c oxidase (CCO) activity while the integrity of the outer mitochondrial from the succinate:cyt c xidoreductase activity, as described by Jiménez et al. (1997). Marker enzymes as lactate dehydrogenase (LDH), NADP-glyceraldehyde 3-P dehydrogenase (G3PDH) and catalase (CAT) were used to check the cytoplasm, chloroplast and peroxisomal

contamination respectively, and they were assayed following *Jiménez et al.* (1997) to assess the purity of the mitochondria.

SOD isozymes were separated by PAGE on 12% gels according to Hernández et al. (1994). Ascorbate peroxidase (APX; EC 1.11.1.11) using its inhibitor pCMS (0.5 mM), monodehydroascorbate reductase (MDHAR; EC 1.1.5.4), dehydroascorbate reductase (DHAR; EC 1.8.5.1) and glutathione reductase (GR; EC 1.6.4.2) activities were assayed according to Jiménez et al. (1997).

2.7. Respiration and oxygen-isotope fractionation measurements

For respiratory measurements, 21-day old plants were placed for 30 min in the dark to avoid light-enhanced leaf dark respiration. Plantlets were carefully harvested from petri dishes and immediately placed in a 3 mL stainless-steel closed cuvette maintained at a constant temperature of 25ºC. Air samples of 250 μL were sequentially withdrawn from the cuvette and fed into the mass spectrometer (Delta XPlus, Thermo LCC, Bremen, Germany). Changes in the $^{18}O/^{16}O$ ratios and oxygen concentration were measured to calculate the oxygen-isotope fractionation as described in Del-Saz et al. (2017). The end point fractionation values of both COP (Δ_c=30.4) and AOP (Δ_a=19.8) were taken from Florez-Sarasa et al. (2012). These are needed for the calculation of the electron partitioning to the alternative pathway (τ_a) and the individual activities of the COP (v_{cyt}) and AOP (v_{alt}) as described in Del-Saz et al. (2017). Values presented are the mean ± SE of eight biological replicates.

2.8. Metabolite profiling

Aerial parts of plantlets were sampled after growing for 21 days in plates containing or not 100 mM NaCl, immediately frozen in liquid nitrogen, and stored at -80°C until further analysis. Samples were then grinded in liquid nitrogen and metabolite extractions were performed as described previously (Lisec et al., 2006) using

approximately 50 mg of frozen-powdered tissue. Metabolites were identified in comparison with database entries of authentic standards (Kopka et al., 2005; Schauer et al., 2005). Chromatograms and mass spectra were evaluated by using Chroma TOF 1.0 (Leco, http://www.leco.com/) and TAGFINDER 4.0 software (Luedemann et al., 2008). The relative content of metabolites was calculated by normalization of signal intensity to that of ribitol, which was added as an internal standard. Thereafter, data were normalized to the mean value of *Col-0* plants under control (no added salt) conditions (i.e. the value of all metabolites for *Col-0* under control conditions was set to 1). Values presented are means ± SE of six replicates each corresponding to a pool of about 3-6 plantlets depending on plant treatment.

2.9. 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 Student´s *t*-test ($P<0.05$). IBM SPSS Statistics 20 programm (Statistical Package for Social Sciences, 2011) and JMP®, Version 12.1.0 (SAS Institute Inc., Cary, NC, USA, 1989–2007) were used for the data analysis.

3. Results

3.1. Growth parameters

After 21 days of growth in the absence or presence of 100 mM NaCl, none of the mutant plants revealed any appreciable phenotype when compared to WT plants in control or saline conditions (Fig. S1), and a visible reduction of the growth was observed under salinity in all the lines. However, analysis of growth parameters

revealed some differences in between WT and mutant plants. Under control conditions, fresh weight of rosette leaves and root length (Fig. 1A and 1D) were significantly decreased in KO2 as compared to WT plants while rosette diameter was shorter in the KO1 mutant (Fig. 1B) and KO2 presented higher number of leaves per rosette (Fig. 1C). These and other differences between both mutants presented in this paper could be due in part to the residual *AtTrxo1* gene expression showed by KO2 plants (Ortiz-Espín et al., 2017b). Salinity provoked a significant decrease in all growth parameters in all the genotypes and differences among them were found for the rosette diameter that was shorter in both mutants, and the number of leaves that was higher in the KO2 plants.

3.2. Isolation of mitochondria

The yield of purified mitochondria from plants grown under control and saline conditions was around 10% measuring cytochrome c oxidase activity in the leaf homogenate and in the isolated organelles (Table 1 and 2). Mitochondrial fractions presented low contamination rates as indicated by the activity of markers enzymes cytoplasmic lactate dehydrogenase (0.7-0.6% in control and saline conditions, respectively), chloroplastic NADP-gliceraldehyde 3-P dehydrogenase (0.01-0.3%) and peroxisomal catalase (1.5-3%). Integrity of isolated mitochondria was found to be around 90%-85% for the organelles isolated from plants grown under control and saline conditions, respectively.

3.3. Mitochondrial oxidative stress status in trxo1 mutants under saline conditions

The analysis of parameters indicative of oxidative stress in isolated mitochondria revealed that the lipid peroxidation (Fig. 2A) was significantly higher in KO2 mutant than in WT plants only under control conditions and salinity did not modify this parameter in any genotype. On the other hand, similar levels of carbonyl (CO) protein groups were found among genotypes in both conditions and salinity only decreased them in the KO2 mutant (Fig. 2B). A similar pattern was found for the H_2O_2 foliar content in the plants, but in this case, KO1 plants showed a significant decrease under salinity (Fig 2C).

3.4. Mitochondrial antioxidant enzymes in trxo1 mutants under saline conditions

Mn-SOD activity was measured after native PAGE in purified mitochondrial fractions (Fig. 3A) from leaves. No significant changes were found among genotypes in control or saline conditions, and only a decrease in the KO2 line with salinity was observed. Catalase activity was measured in foliar extracts with no differences among genotypes in both conditions and the activities were similar between control and saline conditions (Fig. 3B). Measurement of the ASC-GSH cycle enzymes revealed that PCMS-inhibited APX activity was significantly lower in KO2 line that in WT in both control and saline conditions (around 45%) (Fig. 4A). Salinity did not cause a significant change of this activity in any of the lines. The oxidized forms of ASC are regenerated by two enzymes of the ASC-GSH cycle, MDHAR and DHAR. In isolated mitochondria, MDHAR activity was similar in all genotypes under control conditions (Fig. 4B) and was found higher in WT than in KO lines under salinity. Only in WT, salinity provoked an increase in this activity. DHAR activity was similar in all genotypes in both growth conditions with no significant changes provoked by salinity (Fig. 4C). GR activity, the last component of the ASC-GSH cycle involved in the regeneration of GSH from GSSG was higher in KO plants than in WT under control conditions while salinity induced this activity significantly only in the WT genotype (Fig. 4D).

3.5. Respiration and electron partitioning between AOX and COX pathways in trxo1 mutants under saline conditions

Respiratory rates and oxygen isotope fractionation were determined in plants grown under control and saline conditions. Total oxygen uptake (V_t) was not significantly different among lines exposed to either control or saline conditions (Fig. 5A). By contrast, salinity significantly increased V_t (by 23%) only in WT plants. In salt treated plants, τ_a was decreased by 64%, 37% and 30% in WT, KO1 and KO2 plants respectively, relative to control plants, and it remained significantly higher in KO2 line relative to WT plants. Similarly, v_{alt} was significantly decreased in WT, KO1 and KO2 under salinity by 57%, 37% and 30% respectively, relative to control plants. On the other hand, v_{cyt} was only increased in WT plants (by 74%), relative to control conditions Noticeably, the electron partitioning to the alternative pathway (τ_a) was significantly lower in KO1 (35%) and KO2 (27%) lines than in WT plants under control conditions

(Fig. 5B). This was mainly due to a significantly higher activity of the cytochrome pathway (v_{cyt}) displayed by KO1 (36%) and KO2 (29%) (Fig. 5C).

3.6. Immunodetection and redox state of AOX in trxo1 mutants under saline conditions

Enriched mitochondrial fractions were obtained with the addition of NEM to the different preparations, in order to block the Cys residues of the AOX protein. NEM treatment prevents the formation of the disulfide bond between Cys from the AOX monomers and thus, the oxidation of the protein during the mitochondrial enrichment procedure may be avoided. Under these conditions, the relation between the dimeric (oxidized, aprox. 70 kDa) and the monomeric (reduced, aprox 35 kDa) form can be interpreted as the redox state of the AOX protein. No visible band corresponding to the dimeric/oxidized form (aprox 70 kDa) was detected (data not shown) while two major bands corresponding to monomeric forms were detected at about 35 and 39 kDa probably corresponding to different isoforms of the AOX reduced form (Fig. 6). The signal intensities of both 35 and 39 kDa bands (Fig. 6A and 6B, respectively) were similar among genotypes in each of the conditions analyzed. This denotes that *trxo1* mutants do not present significant changes in the AOX protein amount per mitochondria as compared to WT plants. By contrast, the intensity of the 35kDa band was increased under salinity while the one of the 39kD decreased by an approximately similar level in all genotypes.

In order to analyze changes in AOX protein at the leaf level, total leaf extracts were used to immunodetect AOX (Fig. 7). As observed in the mitochondrial enriched fractions, two bands of aprox 35 and 39 kDa were detected. In this case, KO2 leaves displayed a lower amount of the 35 kDa band under control conditions as compared to the other genotypes. The intensities of the 39 kDa bands were similar in all genotypes in both control and saline conditions. These results suggest that KO2 plants may be limited in their AOX capacity for respiration at the leaf level under control conditions. Finally, the signal intensity of the 35 kDa band increased only in the KO2 after salinity treatment while no changes were detected in the other genotypes.

3.7. Metabolite profiles of trxo1 mutants under saline conditions

In order to obtain a more detailed characterization of the metabolic changes linked to the responses of respiration and antioxidant system in the mutants, GC-TOF-MS metabolite profiling analysis was performed in the mutants and WT plants under control and saline conditions. A total of 46 metabolites were annotated after GC-TOF-MS analyses (Supplemental Table S1) and their relative levels were normalized to the mean levels of WT plants under control conditions. Salt treatment significantly ($P<0.05$) decreased the levels of 10 metabolites in WT plants including organic acids such as dehydroascorbate (6.0-fold), fumarate (5.9-fold), malate (3.8-fold), phosphoric acid (2.3-fold) and succinate (1.8-fold); sugars such as glucose (9.4-fold) and fructose (8.3-fold); and amino acids such as glycine (4.0-fold), threonine (1.7-fold) and aspartate (1.3-fold) (Fig. 8 and Supplemental Table S2). By contrast, six metabolites were significantly ($P<0.05$) higher in salt-treated WT plants including trehalose (7.0-fold), raffinose (2.7-fold), proline (2.6-fold), serine (2.0-fold), histidine (1.7-fold) and glycerol (1.34-fold) (Fig. 8 and Supplemental Table S2). With the exception of trehalose under salinity, all the metabolic differences between mutants and WT were observed under the control condition; with both mutant lines consistently displayed lower levels of glucose, fructose, *myo*-inositol and β-alanine while serine and sucrose levels were significantly lower only in the KO2 mutant.

4. Discussion

4.1. Growth and oxidative stress responses of the trxo1 mutants

Salinity stress provokes complex changes in the physiology and metabolism of plants trying to cope with the associated stresses such as ionic, osmotic, oxidative or nitrosative stress. Changes are dependent on many factors including the stress intensity and duration, tissue and development stage, but also the inherent capacity of the plants to sense, cope with or induce defense mechanisms ameliorating deleterious effects (Bartels and Sunkar, 2005; Lázaro et al., 2013). Antioxidant and Trx/Prx systems have been described to play a role in the response to salinity, and specifically Trx*o*1 was

suggested to protect mitochondria from oxidative stress most probably through the regulation of target proteins involved in several processes in the organelle (Martí et al., 2009, 2011; Sevilla et al., 2015a). In the present study, we subjected WT and *trxo1* mutant plants to a long-term salinity treatment by growing them on agar containing 100 mM NaCl. This salinity treatment significantly reduced the fresh weight of all genotypes as previously observed in the same mutants plants grown in soil and exposed to a short-term (7 days) salinity treatment of 100 mM NaCl (Calderón et al., 2018). Differently from Calderón et al. (2018), the KO2 mutant displayed a reduction in both fresh weight accumulation and root length under control conditions and a higher number of leaves; however, these differences could be due to the different growth conditions applied. When grown in soil, salinity induced a lower water loss and higher stomatal closure, H_2O_2 and lipid peroxidation levels in these KO mutants. These effects were accompanied by higher activity of some components of the antioxidant system such as catalase and SOD at the leaf level, probably as a way to compensate the lack of Trx*o*1 (Calderón et al., 2018). In the present study, the leaf contents of H_2O_2 were not increased under salinity in any of the genotypes suggesting the oxidative stress of these plants is milder. Again the different growing conditions and also the duration of the stress applied very likely have influenced on the differences observed with previously obtained results (Calderón et al., 2018).

In order to better understand the responses to salinity at the mitochondrial level, oxidative stress parameters were determined in isolated mitochondria of the *trxo1* mutants. The lack of Trx*o*1 did not result in an important oxidative organelle damage although KO2 mutants presented a different behavior, with higher lipid peroxidation, evidencing its higher sensitivity. Salinity on the other hand did not provoke significant changes on the mitochondrial oxidative stress parameters probably due to the response of an efficient mitochondrial antioxidant system.

4.2. Mitochondrial antioxidant systems responded differently in trxo1 mutants

The mitochondrial respiratory chain is a source of ROS and the presence of a ROS scavenging system in the organelle allows it to cope with the oxidative stress induced

mainly under stress situations. For this reason, components of the ASC-GSH cycle collaborate with Mn-SOD and the Trx/Prx system present in this cellular compartment. Previously reported changes of the antioxidant and redox systems denote that stress tolerance likely requires the induction of specific isoforms in the different cell compartments or a constitutively higher content of antioxidants, depending on the species, variety or strength and duration of stress (Lázaro et al., 2013; Hossain and Dietz, 2016).

An increase in mitochondrial Mn-SOD activity and RNA expression have reported in a salt tolerant but not in sensitive pea cultivars, being these inductions dependent on the salt concentration (Gómez et al 1999; Hernández et al., 2000), and sometimes the existence of post-transcriptional regulation has been suggested to explain a maintained Mn-SOD protein during the stress in those tolerant pea cultivars showing a high Mn-SOD activity (Camejo *et al*., 2013). Likewise the overexpression of Mn-SOD increased tolerance to salinity in different plants such as Arabidopsis, tomato and poplar (Wang et al., 2004, 2007, 2010). More recently we have found an increase in all the SOD isoenzymes in soil-grown Arabidopsis plants under salinity which was more pronounced in *trxo1* mutants than in WT plants and coincided with increased H_2O_2 content in leaves (Calderón et al., 2018). The fact that neither mitochondrial Mn-SOD nor CAT activities were responding to the lack of Trx*o*1 or salinity in the present study (Figure 3) points to compensating events occurring in the KO mutants in both control and saline conditions when grown on plates, which finally allowed them to keep the H_2O_2 levels constant (Figure 2). When the oxidative stress indicators were analysed in mitochondria, neither protein oxidation nor lipid peroxidation increased under salt treatment, sustaining that plants are not subjected to a severe oxidative stress under these saline conditions. On the other hand, no changes in Mn-SOD and CAT activities were previously reported in Arabidopsis and pea plants under salinity or other environmental stresses (Kliebenstein et al., 1998; Martí et al., 2011; Wang et al., 2004), in which a mild oxidative stress was induced. That pattern seems to be very similar to that observed in our study either in *trxo1* mutants as in WT plants.

In this scenario, we have also examined the mitochondrial ASC-GSH cycle enzymes as another important antioxidant system regulating the endogenous levels of H_2O_2 very effectively (Jiménez et al., 1997; Chew et al., 2003). In a previous study using the same Arabidopsis genotypes, we described that total leaf APX activity

increased under salinity in both WT and KO *trxo1* mutants (Calderón et al., 2018). Studies at the leaf level have reported controversial results with regard to APX activity (Wang et al., 2013). Our aim in the present study was to focus on the effects of the *trxo1* mutation on the ASC-GSH cycle at the mitochondrial level in which the information is quite reduced. The presence of APX in mitochondria was previously reported in both pea leaves and potato tubers (Jiménez et al., 1997; De Leonardis et al., 2000). In Arabidopsis, APX is dually targeted to mitochondrial and stroma compartments (Chew et al., 2003). In our study, the lower mitochondrial APX activity regulating H_2O_2 in the KO2 mutant could have an impact in the observed oxidation of lipids in these plants, without eliminating the possibility of contribution of other antioxidants as PrxIIF also controlling peroxide levels. On the other hand, the activity of the mitochondrial APX was not significantly changed in WT plants under salinity, while the activities of mitochondrial MDHAR and GR were increased (Figure 4). This pattern of change in MDHAR and GR activities is similar to that reported in mitochondria from salt-tolerant tomato cultivars and relatively salt-tolerant pea plants, although in pea, APX activity also increased under mild and severe salt stress (Gómez et al., 1999; Mittova et al., 2003; Lázaro et al., 2013).

In the *trxo1* mutants, none of the activities of the mitochondrial components of the ASC-GSH cycle enzymes were induced by the salinity treatment (Figure 4). Notably, the mitochondrial activity of GR was higher in both mutants than in the WT plants under control conditions and it was not diminished upon salinity. This observation, together with the maintained levels of DHAR activity could have influenced the glutathione and ascorbate recycling and the level of H_2O_2. As mentioned above, specifically under salinity, increases in the GR activity have been positively correlated to salinity tolerance (Lázaro et al., 2013). Furthermore, the overexpression of GR has been described to increase tolerance of tobacco and poplar plants to salinity finding a higher content of ASC in the leaves (Aono et al., 1993; Foyer et al., 1995). In this way, the lack of Trx*o*1 could be compensated by higher GR activity under control conditions in the mutants, and the maintenance of that constitutive level in salinity contributed to an efficient response of these plants to the stress condition. An inverse relationship between Trx and GSH has also been described in over-expressing PsTrx*o*1 tobacco BY-2 cells, in yeast *trx1-trx2* double mutant and in an Arabidopsis GSH-deficient

meristemless mutant, as a mechanism to adjust GSH levels (Ortiz-Espín et al., 2015; Schnaubelt et al., 2015; Calderón et al., 2017).

4.3. The alternative oxidase (AOX) pathway is differentially regulated in trxo1 mutants

An induction of the *in vivo* AOX activity under salinity has only been observed after a short-term and severe stress (Del-Saz et al., 2016) while long-term either decreases in the case of very severe stress (Del-Saz et al., 2016), or maintains the *in vivo* AOX activity (Martí et al., 2011). In the present study, a long-term salinity stress was applied in plants grown in agar plates that, as previously discussed, only caused a mild oxidative stress. Under this condition, the AOX displayed a reduction of its *in vivo* activity in all genotypes, suggesting that it was probably not required because of a stress-adaptation of the plants. Nevertheless, the higher electron partitioning to the AOX pathway under salinity observed in both mutants (Figure 5B) denotes a relatively higher response of the AOX that can act preventing the generation of the radical superoxide at the UQ level (Purvis, 1997; Cvetkovska and Vanlerberghe, 2012), with a possible influence in the response of the enzymatic system controlling H_2O_2 levels in these plants.

The lack of the proposed activator of the AOX, the Trx*o*1, was expected to cause a decrease in the reduction level of the AOX and thus limit its activation under saline condition. In order to further investigate this, we determined the redox state of the AOX protein in enriched mitochondrial fractions (Figure 6). Unlike in leaf extracts, the AOX protein in mitochondria isolated from Arabidopsis, pea and tobacco has been reported predominantly in its dimeric-oxidized form (Rhoads et al., 1998; Martí et al., 2009; Vanlerberghe, 2013), very likely due to its oxidation during the isolation procedure (Umbach and Siedow, 1997; Nietzel et al., 2017). The use of a chemical crosslinker such as NEM during the mitochondrial isolation or enrichment procedure should have avoided the formation of the dimeric forms thus approximating the *in vivo* redox state situation. By using this technique, no apparent changes on the AOX redox state were detected in the *trxo1* mutants as compared to WT plants. Moreover, the AOX was invariably found in its reduced monomeric state not only under salinity but also under

control conditions. These results do not allow us to establish a lack of direct redox regulation of AOX by the Trx*o*1 *in vivo*, because of the presence of other systems involving other thioredoxins or thiol reductases as glutaredoxins (Meng et al., 2010; Moseler et al., 2015; Ströher et al., 2016), which could perform a maintenance function, avoiding any oxidation or reversing it efficiently, rather than an AOX regulation function. This possibility has been suggested recently to explain the function of mitochondrial Trx on AOX (Nietzel et al., 2017). In this case, the constitutive GR activity in the mutants, at higher and/or similar levels to those of the WT genotype, could facilitate the necessary GSH for the reduction of GRXs, something that deserves further investigations. Nevertheless, our results are in the line of several previous studies suggesting that the AOX protein is mainly found in its reduced state and therefore it´s *in vivo* activity is probably mainly regulated by the mechanism involving organic acids interaction and by its substrate availability (Del-Saz et al., 2018).

The presence of different bands in our western blot analysis is similar to that described by several authors in different species including Arabidopsis (Umbach et al., 2005; Djajanegara et al., 2002). Simons et al. (1999) described the existence of different protein bands possibly as a result of the multigene family of AOX in Arabidopsis (Saisho et al., 1997) as it was observed in soybean (Whelan et al., 1996). Also Wanniarachchi et al. (2018), has recently described two monomeric forms that may reflect two different genes or as described in soybean, different initiation of translation of an AOX gene. These authors described the tissue- and developmental stage-dependent control of the different AOX genes expression, also responding to different environmental signals and generating several AOX protein bands on immunoblots (Tanudji et al., 1999). Regardless of the redox state, the KO2 mutant displayed a different pattern on the total AOX protein amount detected in whole leaf extracts that is similar to its pattern of *in vivo* partitioning to the AOX. This suggests that the *in vivo* AOX activity in this mutant may be operating at its full capacity and thus depends on the protein amounts, i.e. is limiting under control conditions and then increased under salinity, as compared to WT plants (Figures 5 and 7). The reason for this altered pattern on the expression of the AOX and its possible full activation remains elusive. However, an indirect effect of the lack of Trx*o*1 on the *in vivo* activity of the AOX may be related to metabolic regulation. In this respect, the Trx*o*1 has been shown to regulate the carbon flux into the TCA cycle (Daloso et al., 2015) which can affect the level of organic acids

and mitochondrial NADH production which both in turn affect the AOX activity (Selinski et al., 2017; Del-Saz et al., 2018). Organic acids such as oxaloacetate and 2-oxoglutarate can stimulate differently AOX1a, AOX1c and AOX1d isoforms while pyruvate is able to activate all of them (Selinski et al., 2018). The fact that our *trxo1* mutants did not present changes in pyruvate (Figure 8) but showed altered expression of different isoforms may point to a possible differential regulation by other organic acids. The presence and induction of different AOX isoforms in our *trxo1* mutants may be due to differential regulation of gene expression. It has been shown that AOX1a is the predominant form in Arabidopsis being highly inducible by many treatments, while AOX1c is widely expressed but its expression did not change with oxidative stress-induced treatments (Thirkettle-Watts et al., 2003; Clifton et al., 2005; Elhafez et al., 2006). Also the different behavior of the isoforms could be due, as mentioned by Selinski et al. (2018), to differences between homodimers and heterodimers of AOX, something that would merit a deeper *in vitro* and *in vivo* investigation.

4.4. Respiratory carbon and energy metabolism in the trxo1 mutants

Mitochondrial Trx*o*1 has been shown to deactivate mitochondrial succinate dehydrogenase and fumarase and activate the cytosolic ATP-citrate lyase, thus regulating carbon flow through the TCA cycle (Daloso et al., 2015). In fact, some TCA cycle intermediates accumulate in *trxo1* mutant plants grown in soil under a short photoperiod (Daloso et al., 2015). We did not detect significant changes in organic acids levels but we clearly observed pronounced changes on the levels of sugars in the *trxo1* mutants under both control and saline conditions, thus also indicating a reorganization in central carbon pathways. A dramatic decrease on glucose and fructose levels was observed in both *trxo1* mutants as compared to WT plants. This could indicate a higher use of these sugars in the glycolytic pathway that would cause an increased respiration, probably driven by an increased carbon flow through the TCA cycle as previously suggested (Daloso et al., 2015). In agreement, higher *in vivo* respiratory activity through the COX pathway was observed in the mutants (Figure 5). This increase in ATP-coupled respiration can also indicate an increase on the leaf energy demand on the mutants under control conditions.

Recently, new hypotheses have postulated that sucrose is a key regulator of stomatal movement (Lima et al., 2018). During stomatal opening sucrose can serve as an energy source in the guard cells through its oxidation by glycolytic and TCA cycle metabolism (Medeiros et al., 2018). Curiously, leaves of *trxo1* mutants display higher number of stomata than WT (Calderón et al., 2018) thus potentially requiring a higher respiratory flux at the leaf level. Therefore the lower levels of sucrose, glucose and fructose observed here in the *trxo1* mutants can be explained as a result of their higher oxidation by the glycolysis and TCA cycle activity coupled to ATP production by COX activity. This high respiratory cost could be the cause for a more negative carbon balance in the *trox1* mutants thus explaining its reduced growth as compared to WT plants under control conditions. Under saline conditions, the levels of sugars decreased probably because of a reduced photosynthetic activity typically observed under this condition (Martí et al., 2011) and thus cancelling the observed metabolic phenotype of *trxo1* mutant plants. Nevertheless, the opposite pattern of the levels of trehalose observed in WT and mutant plants probably reflects changes on sugar sensing, metabolism and stomatal regulation (Lunn et al., 2014) as a consequence of the disturbed sugar metabolism in the *trxo1* mutants. Future experiments will be required to unravel the precise mechanisms that involve the complex interplay between respiration, sugar metabolism and stomatal aperture.

5. Conclusions

In summary, our results showed that *trxo1* mutants displayed an acclimation to mild salt stress similar to WT plants. This was favored by a coordinated response involving changes of several antioxidant systems, sugars metabolism and energy efficiency of respiration through the regulation of target proteins involved in these processes, in this way diminishing oxidative stress. However, future experiments under different salinity conditions are needed to determine whether this response can be considered as a typical mechanism to compensate for a lack of Trx*o*1.

Declaration of interest

None.

Author contribution

AJ, MRC and FS designed the research. ASG, NFD-S, and IFS performed the research. AJ, FS, AF and IFS analyzed the data and results. AJ, IFS and FS wrote the manuscript.

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Supplementary material

Supplementary material can be found online**.**

Fig. S1. Representative 21-day-old Arabidopsis WT and two KO *AtTrxo1* mutant lines (KO1 and KO2) growing in the absence (control) or presence of 100 mM NaCl.

Table S1. Parameters used for peak annotation in GC-MS analysis

Table S2. Relative metabolite levels in Col-0 WT and two KO *AtTrxo1* mutant lines grown in the absence (C) and presence of 100 mM NaCl (S). Data are presented as mean ± SE for six biological replicates normalized to the mean level of the WT plants under control conditions. Bold numbers denote significant differences ($P < 0.05$) to the control conditions in each genotype, and asterisks indicate significant differences ($P < 0.05$) to the WT in each condition.

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Tables

Table 1. Distribution of marker enzymes in different subcellular fractions from the aerial part of 21-old Arabidopsis wild type (WT) plants grown in control conditions.

	Distribution of total enzyme activity (%)			
	CCO	LDH	G3PDH	CAT
Crude extract	100	100	100	100
12,000g pellet	16	11	0.3	29
Isolated mitochondria	10.5	0.7	0.01	1.5

Table 2. Distribution of marker enzymes in different subcellular fractions from the aerial part of 21-old Arabidopsis wild type (WT) plants grown in saline (100 mM NaCl) conditions.

	Distribution of total enzyme activity (%)			
	CCO	LDH	G3PDH	CAT
Crude extract	100	100	100	100
12,000g pellet	24	14.3	1.1	48
Isolated mitochondria	9.5	0.6	0.3	3

Figure legends

Fig. 1. Growth parameters (A) plant fresh weight, (B) rosette diameter, (C) number of leaves and (D) root length of *Col-0* WT and two KO *Attrxo1* mutant lines 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 student t-test (P <0.05).

Fig. 2. Oxidative parameters. (A) Mitochondrial malondialdehyde (MDA), (B) mitochondrial carbonyl (CO) proteins and (C) foliar hydrogen peroxide per fresh weight (FW) contents of *Col-0* WT and two KO *Attrxo1* mutant lines 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 student *t*-test (P <0.05).

Fig. 3. SOD and catalase activities. (A) Mn-SOD activity after PAGE in mitochondrial-enriched fractions and (B) foliar catalase activity in *Col-0* WT and two KO *Attrxo1* mutant lines 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 student *t*-test (P <0.05).

Fig. 4. Ascorbate-glutathione cycle enzymatic activities. (A) Ascorbate peroxidase APX, (B) monodehydroascorbate reductase MDHAR, (C) dehydroascorbate reductase DHAR and (D) glutathione reductase GR in *Col-0* WT and two KO *Attrxo1* mutant lines 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 student *t*-test (P <0.05).

Figure 5. *In vivo* mitochondrial electron transport chain activities. (A) Total respiration (V_t), (B) electron partitioning to the alternative pathway (t_a), (C) cytochrome pathway activity (v_{cyt}) and (D) alternative pathway activity (v_{alt}) in plantlets of *Col-0* (WT) and two KO *Attrxo1* mutant lines grown in the absence (control) and presence of 100 mM NaCl. Values are means ± SE of 8 biological replicates. 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 student t-test (P <0.05).

Fig. 6. Immunoblotting of AOX protein in enriched-mitochondrial fractions of *Col-0* WT and two KO *Attrxo1* mutant lines grown in the absence (control) and presence of 100 mM NaCl. Mitochondrial proteins were separated by SDS–PAGE, immunoblotted with monoclonal AOX antibody and visualized using the ECL Plus Western blotting detection system, as described in Materials and Methods. Quantification of the two bands around 35 (A) and 39 kDa (B) was performed using the Quantity One software-based analysis (BioRad). 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 student *t*-test ($P < 0.05$).

Fig. 7. Immunoblotting of AOX protein in total seedling extracts of *Col-0* WT and two KO *Attrxo1* mutant lines grown in the absence (control) and presence of 100 mM NaCl. Proteins were separated by SDS–PAGE, immunoblotted with monoclonal AOX antibody and visualized using the ECL Plus Western blotting detection system, as described in Materials and Methods. Quantification of the two bands around 35 (A) and 39 kDa (B) was performed using the Quantity One software-based analysis (BioRad). 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 student *t*-test ($P < 0.05$).

Figure 8. Heat map showing the relative levels of the metabolites in Col-0 WT and two KO *Attrxo1* mutant lines grown in the absence (C) and presence of 100 mM NaCl (S). Metabolites were clustered per class into amino acids, organic acids, sugars and sugar alcohols, and other metabolites. Relative metabolite levels in leaves of all lines under both control and salinity conditions were normalized to the mean level of the WT plants under control conditions and fold-change values were log2 transformed (i.e. the level of all metabolites of WT plants under control is 0). In this heat map, red and blue colors represent log2 fold-increased and -decreased metabolites, respectively. Values are means ± SE of six replicates and asterisks denote significant differences ($P < 0.05$) to the WT plants in each condition. The statistical differences between control and salinity treatments in each genotype are presented in Supplemental Table S2.