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Salinity and osmotic stress trigger different antioxidant responses related to cytosolic ascorbate peroxidase knockdown in rice roots

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ABSTRACT

Salinity and osmotic stress trigger distinct signals in roots, which might induce differences in antioxidant responses. To clarify these relationships, transgenic rice plants silenced in both cytosolic ascorbate peroxidases (*apx1/2*) and non-transformed (NT) were exposed to iso-osmotic concentrations of NaCl and mannitol. Under both stress conditions, *apx1/2* roots did not suffer oxidative stress, revealing that cytosolic APXs were not crucial to oxidative protection. Silenced and non-transformed roots triggered different responses to high salinity and osmotic stress and these stressful factors induced also distinct antioxidant changes. High salinity up-regulated expression of important *OsAPX* isoforms and these changes were related to increased APX activity, especially in NT roots. Intriguingly, salt stress triggered up-regulation of *OsCAT* isoforms but CAT activity did not change in both genotypes. In contrast, mannitol trigged very low increment in expression of *OsAPX* isoforms but induced substantial up-regulation in APX activity in NT roots. Mannitol also remarkably up-regulated *OsCATB* expression in parallel to CAT activity, in both *apx1/2* and NT roots. POD and GPX (glutathione peroxidases) activities were strongly increased by high salinity but did not change in response to mannitol, in both genotypes. The two stress types as well as *apx1/2* and NT roots displayed different response in terms of modulation in the H_2O_2 levels but lipid peroxidation did not change. Membrane integrity was drastically affected by both stressful factors and similarly in both genotypes, whereas root fresh matter was affected only by salt stress. Altogether, the obtained data reveal that high salinity and osmotic stress trigger different antioxidant responses and these strategies were genotype-dependent. The different antioxidant molecular-biochemical mechanisms employed by cytosolic APX knockdown and non-transformed roots allowed reaching similar physiological performance.

1. Introduction

Plant roots play critical roles during growth and development, such as mineral nutrient uptake, maintenance of adequate plant water balance and hormone synthesis (Pierik and Testerink, 2014). Moreover, roots are intrinsically related to stress perception and signaling mechanisms connecting soil factors with whole plant metabolism (Choi et al., 2014). Soils are important sites of specific abiotic stresses, such as water deficit, high salinity, toxicity, nutrient deficiency, flooding etc. (Gupta et al., 1999; Małecka et al., 2001; Zhang and Zhang, 1994; Tabata et al., 2014). Most of these stressing factors have as a common characteristic the generation of a secondary stress related to disturbances in the redox metabolism (Adem et al., 2014). This secondary oxidative stress in roots is resultant from an unbalance between production and scavenging of reactive oxygen species (ROS) (Adem et al., 2014; Cavalcanti et al., 2007).

The mechanisms involved with generation and elimination of ROS in roots under high salinity and osmotic stress are scarcely known (Adem et al., 2014; Cavalcanti et al., 2007; Hernandez et al., 1993). High salinity has two components; ionic toxicity and osmotic and these elements might induce different types of responses in roots (Flowers et al., 2015; Munns and Gilliham, 2015;

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Roy et al., 2014). Both saline and osmotic agents might generate disturbances in root metabolism, including deleterious effects in several organelles and cellular compartments (Flowers et al., 2015; Zhu, 2002). These alterations might affect especially the cell wall, apoplast, cytosol and mitochondria, inducing changes in gene expression and enzyme activities related to redox metabolism (Maia et al., 2013). Under these conditions, frequently the production of ROS is over increased and the antioxidant mechanisms might be insufficient to re-establish a favorable redox homeostasis (Foyer and Noctor, 2015).

The molecular-biochemical mechanisms involved with root perception of signals from saline ions and osmotic stress are complex and still poorly understood (Choi et al., 2014). After perception and signal transduction in cytosol, a cascade of reactions occurs until the expression of different stress-related genes (Maathuis, 2014). These genes encode for several important proteins including those associated with root growth and ROS scavenging (Mittova et al., 2004). ROS production in roots might occur from biochemical and chemical processes such as Haber-Weiss-Fenton reactions (Halliwell and Foyer, 1976), excess energy in mitochondrial electron transport chain – ETC (Møller, 2001), up-regulation of NADPH oxidase in plasmalemma (Passardi et al., 2004) and alterations in the cytosolic ascorbate-glutathione cycle (Munné-bosch et al., 2013).

Salt and osmotic stresses might induce down- or up-regulation in the expression of genes that encode for catalases (CAT), ascorbate peroxidases (APX) and glutathione peroxidases (GPX) and type III peroxidases (POD) (Hong et al., 2009, 2007; Mittova et al., 2004). APX and CAT are the most important peroxidases responsible for scavenging and maintaining of H_2O_2 in levels adequate to redox homeostasis in roots (Cavalcanti et al., 2007). Rice has eight APX isoforms, viz. two localized in mitochondria, two in cytosol, two in chloroplast and two in peroxisomes (Teixeira et al., 2006, 2004). The cytosolic APX isoforms in plants are found in high concentrations and these enzymes are strongly involved in protection against abiotic stress in leaves (Shigeoka and Maruta, 2014). These enzymes are the most important members of APX family in antioxidant protection (Shigeoka and Maruta, 2014).

The role and importance of cytosolic APX isoforms as well as its relationships with other peroxidases in root redox metabolism is incipient (Maia et al., 2013). The antioxidant metabolism in roots, especially in response to ionic toxicity and osmotic stress, is much less studied compared to leaves. The majority of published articles concerning the saline and osmotic stress responses in roots are descriptive and the underlying mechanisms involving the role of each specific antioxidant are poorly understood (Cavalcanti et al., 2007; de Azevedo Neto et al., 2006; Maia et al., 2013). For instance, which is the importance of cytosolic APXs and CAT in scavenging and maintaining of H_2O_2 homeostasis in roots exposed to acute salinity and osmotic stress? In addition, which is the role, complementary or primary, of other peroxidases such as CAT, POD and GPX in such processes? Undoubtedly, the knowledge on redox metabolism generated from leaf studies would not be extensible for roots since that these organs present very different structures related to oxidative and antioxidant metabolism.

We hypothesized here that high salinity and osmotic stress trigger different antioxidant responses in rice roots and these differences also are distinct among cytosolic APXs deficient (knockdown) and non-transformed rice roots. This study revealed that ionic and osmotic stress triggered very different antioxidant responses represented by the differential modulation in *OsAPX* and *OsCAT* expression and regulation of APX, CAT, GPX and POD activities. The role of H_2O_2 in these responses and the physiological significance in terms of oxidative in roots is discussed.

2. Materials and methods

2.1. Construction of the plant vector and plant transformation

The non-transformed (NT) and transgenic (*apx1/2*) rice (*Oryza sativa* L. cv. Nipponbare) plants were obtained as previously reported by Rosa et al. (2010). Chimerical gene producing mRNA with a hairpin structure (hpRNA) was constructed based on the sequence of the *OsAPX1* (LOC_Os03g17690) and *OsAPX2* (LOC_Os07g49400) genes. The following primer pairs were used: CGCCGCCAACGCCGGCCTCGA and CACTCAAACCCATCTGCGCA (*OsAPX1/2*RNAi). PCR products were cloned into the Gateway vector (pANDA), in which hairpin RNA is driven by a maize ubiquitin promoter and an intron that is placed 50 bp upstream of inverted repeats (Miki and Shimamoto, 2004). Agrobacterium mediated transformation was performed as described previously (Rosa et al., 2010). After an initial screening involving 15 lines, three were selected (*apx1/2*-5, *apx1/2*-10 and *apx1/2*-11). These lines showed similar molecular and physiological characteristics (Rosa et al., 2010). Previously (Bonifacio et al., 2011), and in this study, the *apx1/2*-5 line was used, at the F3 generation, as a representative mutant of the *apx1/2* double-silenced lines. These plants have exhibited a similar response in terms of *OsAPX1/21* and *OsAPX2* transcript amount and APX activity in leaves at F1, F2 and F3 generations (Bonifacio et al., 2011).

2.2. Plant growth and treatments

apx1/2 and the NT seedlings (7-day-old) were transferred to 3 L plastic pots filled with half-strength Hoagland-Arnon's nutritive solution (Hoagland and Arnon, 1950). The pH was adjusted to 6.0 ± 0.5 every two days, and the nutrient solution was changed weekly. The plants were grown for 45 days in a greenhouse under natural conditions as follow: day/night mean temperature of 29/24 °C, mean relative humidity of 68%, and a photoperiod of 12 h. The light intensity inside the greenhouse varied as a typical day from 6:00 a.m. to 6:00 p.m., reaching an average of maximum PPFD equals to 820 $\mu mol\ m^{-2}\ s^{-1}$ at noon. NT and *apx1/2* plants were grown in nutrient solution supplied with NaCl and mannitol, which were used in iso-osmotic concentrations corresponding to −0.62 MPa. The osmolality was measured in a vapour pressure osmometer (Model 5520, Wescor®, USA) and the final concentrations of NaCl and mannitol were adjusted to 150 mM and 268 mM, respectively. The nutrient solution without these two solutes was used as control. NaCl and mannitol were added to the nutrient solution in two steps (half of each solute per day) to avoid osmotic shock. Plants were subjected to these stressful conditions for eight days. Subsequently, 5 cm from the superior part of mature rice roots were immediately harvested, frozen in liquid N_2 and stored at −80 °C until the biochemical and transcript analyses.

2.3. Na^+ and K^+ content determinations

The Na^+ and K^+ contents in roots were determined as previously described (Marques et al., 2013). Lyophilized root samples were transferred to hermetically sealed tubes containing deionized water. Subsequently, the samples were boiled in water bath at 100 °C for 1 h. After extract filtration using filter paper, the Na^+ and K^+ contents were determined by flame photometry (B462, Micronal®, Brazil).

2.4. H_2O_2 concentration, membrane damage (electrolyte leakage) and lipid peroxidation (TBARS content)

Hydrogen peroxide content was measured using the Amplex®-red kit (Thermo Fisher Scientific®, USA), based on colorimetric

measure of resorufin formation in presence of H_2O_2 (Zhou et al., 1997). Fresh root samples were macerated with liquid N_2 in the presence of 100 mM K-phosphate buffer pH 7.5 and centrifuged at 12,000 x *g* (4 °C) during 30 min. The supernatant was immediately used for H_2O_2 determination. The measurements were performed after reading at 560 nm. The hydrogen peroxide content was calculated from a standard curve, and the results were expressed as nmol H_2O_2 g^{-1} fresh weight (FW). Cellular integrity (electrolyte leakage) was measured as described by Blum and Ebercon, (1981). Root samples (middle portion) were placed in tubes containing deionized water. The flasks were incubated in a shaker for 12 h, and the electric conductivity in the medium (L1) was measured. Then, the medium was boiled (95 °C) for 60 min and the electric conductivity (L2) was measured again. The relative membrane damage (MD) was estimated by MD = L1/L2 × 100. Lipid peroxidation was measured based on the formation of thiobarbituric acid-reactive substances (TBARS) in accordance with Cakmak and Horst, (1991). The concentration of TBARS was calculated using its absorption coefficient (155 mM^{-1} cm^{-1}), and the results were expressed as nmol MDA-TBA g FW^{-1}.

2.5. Quantitative real-time RT-PCR (qRT-PCR)

qRT-PCR experiments were carried out using cDNA synthesized from roots total RNA and purified with Trizol (Invitrogen®, USA) as previously described (Rosa et al., 2010). Pairs of primers to amplify the *Osfdh3* gene (LOC_Os02g57040) and the *Osfa1* gene (LOC_Os03g08020) were used as internal controls to normalize the amount of mRNA present in each sample. All qRT-PCR reactions were performed with a StepOne plus™ Real-Time PCR system (Applied Biosystems®, USA) using SYBR-green intercalating dye fluorescence for detection.

2.6. Protein extraction and enzyme activity assays

For preparation of enzymatic extracts, fresh root samples were ground to a fine powder in the presence of liquid N_2 using a mortar and pestle and extracted in ice-cold (4 °C) and 100 mM K-phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 2 mM ascorbic acid (to prevent APX denaturation). The homogenate was centrifuged at 15,000 x *g* for 30 min, and the obtained supernatant was used for determination of all enzymatic activities: catalase (CAT; EC 1.11.1.6); ascorbate peroxidase (APX; EC 1.11.1.11); glutathione peroxidase (GPX; EC 1.11.1.9) and type III peroxidase (POD; EC 1.11.1.7). All steps were performed under low temperature (2–4 °C). The protein content was measured by Bradford method (Bradford, 1976), utilizing BSA as a standard.

CAT activity was measured following the oxidation of H_2O_2 at 240 nm. CAT was determined after the reaction of the enzymatic extract in the presence of 50 mM potassium phosphate buffer (pH 7.0) containing 20 mM H_2O_2. The reaction took place at 30 °C with the absorbance monitored at 240 nm over 300 s (Havir and McHale, 1987). CAT activity was calculated using the molar extinction coefficient of H_2O_2 (40 M^{-1} cm^{-1}) and was expressed as μmol H_2O_2 mg^{-1} protein min^{-1}. APX activity was assayed in a reaction mixture containing 0.5 mM ascorbate and 0.1 mM EDTA dissolved in 100 mM K-phosphate buffer (pH 7.0) and enzyme extract. The reaction started by adding 3 mM H_2O_2. Enzyme activity was measured by following the decrease in absorbance at 290 nm (25 °C) over 180 s (Nakano and Asada, 1981). APX activity was calculated from the ASC molar extinction coefficient (2.8 mM^{-1} cm^{-1}) and activity was expressed as μmol ASC mg^{-1} protein min^{-1}. GPX activity was measured according to the method of Awasthi et al. (1975). To perform the enzymatic assay, the extract were mixed in a reaction medium containing K-phosphate buffer 100 mM, pH 7.0, 4 mM GSH, 0.2 mM NADPH and 0.05 U of Glutathione Reductase (type II from wheat, Sigma-Aldrich®, USA). The reaction was initiated by addition of cumene hydroperoxide. GPX activity was determined by the decrease of NADPH absorption at 340 nm. The non-specific NADPH decrease was corrected using the same medium free of cumene hydroperoxide. The GPX activity was estimated from the molar extinction coefficient of NADPH (6.22 mM^{-1} cm^{-1}) and expressed as nmol NADPH mg^{-1} protein min^{-1}. POD activity was assayed by measuring the rate of guaiacol oxidation using the method of Amako et al. (1994). The reaction medium consisted of 50 mM K-phosphate buffer (pH 7.0), 20 mM guaiacol as an electron donor and 0.1 mM hydrogen peroxide. The reaction product intensity was measured in spectrophotometer at 430 nm. POD activity was estimated utilizing the molar extinction coefficient of tetraguaiacol (26.6 mM^{-1} cm^{-1}) and was expressed as μmol tetraguaiacol mg^{-1} protein min^{-1}.

2.7. Western blotting of cytosolic APX proteins

Root protein extracts were first separated by SDS-PAGE (Laemli, 1970). Equal amounts of protein (20 μg) were electrophoretically transferred to a nitrocellulose membrane (Towbin et al., 1979). Polypeptide detection was performed using specific polyclonal antibodies against APX (Agrisera©, Sweden). Membranes were blocked overnight with 5% non-fat milk in saline Tris-HCl buffer (100 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween-20), incubated with secondary antibodies conjugated with peroxidase (Agrisera©, Sweden) and developed using a chemiluminescent detection ECL standard reagents system® (GE Healthcare, USA). All the procedure was realized according manufacturer's manual.

2.8. Statistical analyses and experimental design

The experiment was arranged in a completely randomized design in a 2 × 3 factorial: two genotypes (NT and *apx1/2*) and three treatments (control, NaCl and mannitol), with four independent replicates. An individual pot containing 2 plants represented each replicate. Data were analyzed using ANOVA, and the means were compared using the Tukey's test at a confidence level of 0.05.

3. Results

3.1. Phenotypic characterization, OsAPX1 and OsAPX2 transcripts, protein abundance and APX activity in silenced roots under control condition

The 45-day-old cytosolic APX silenced rice plants (*apx1/2*) did not exhibit any apparent phenotypic changes in their root morphology and fresh matter, compared to NT plants (Fig. S1A and B). It is important to note that in roots the silencing intensity of both APX1 and APX2 was similar to those previously observed in rice leaves (Carvalho et al., 2014). Indeed, the amount of both *OsAPX1* and *OsAPX2* mRNAs were efficiently targeted by the hairpin construct, drastically reducing their accumulation by approximately 90% (Fig. 1A). In addition, western blotting analysis revealed a complete absence of both APX1 and APX2 proteins compared to NT (Fig. 1B) and APX activity was notably decreased by 66% in relation to NT (Fig. 1C). These results strongly suggest that the two cytosolic APXs played an insignificant importance for

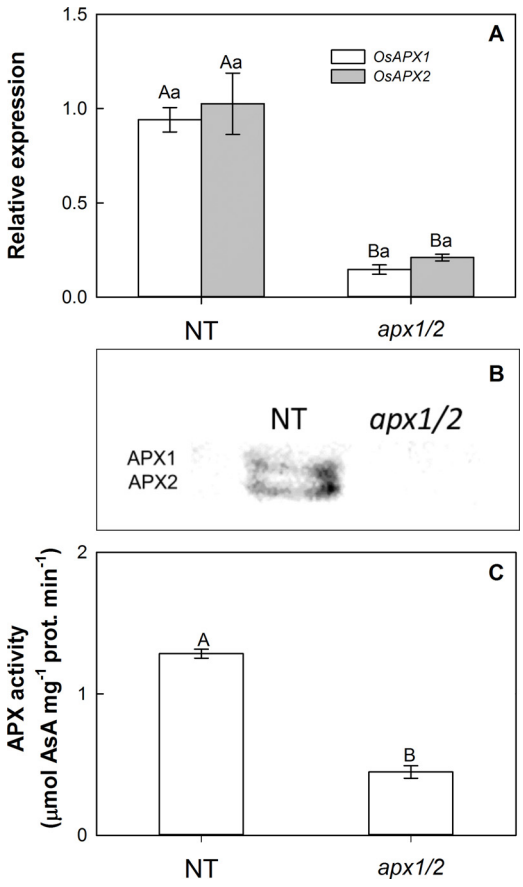

Fig. 1. Transcript levels of *OsAPX1* and *OsAPX2* (A), APX1 and APX2 western blotting (B) and APX activity in roots of NT and *APX1/2* plants grown in nutrient solution for 45 days. Different capital letters represent significant differences among the NT and *apx1/2* plants within different treatments whereas different lowercase letters represent significant differences between treatments, within genotypes. Data are means of four replicates (±SD) and the averages were compared using the Tukey's test, at a confidence of 0.05. The relative expression of each APX locus was normalized by the average value obtained from the NT control plants.

normal growth and development of rice roots under control condition.

3.2. High salinity caused drastic effects on root growth and cellular integrity but these effects were not aggravated by apx1/2 *deficiency*

NT and *apx1/2* plants were exposed to high salinity (NaCl 150 mM) for eight days to examine the cumulative effects induced by osmotic and ionic toxicity components in rice roots. Salt stress caused drastic effects on the growth (fresh matter accumulation) in both shoot (Fig. S1C-D) and roots (Table S1). These detrimental effects produced by NaCl were similar in NT and transgenic plants deficient in both cytosolic APXs. Both studied plants showed high Na^+ content in roots in response to NaCl treatment but these values were higher in silenced rice. The Na^+/K^+ ratios were relatively high in both salt-treated genotypes (from 2.0 in *apx1/2* to 2.5 in NT), indicating the occurrence of osmotic stress followed by Na^+ toxicity in roots (Table S1). Corroborating these results, both genotypes suffered strong and similar increase in root membrane damage (electrolyte leakage), corresponding to an increase of approximately two-fold in relation to control (Table S1).

3.3. High salinity up-regulated transcript amounts of APX1 and APX2, and stimulated APX activity and did not induce oxidative stress in both NT and apx1/2 *roots*

High salinity triggered up-regulation in mRNAs of both APX1 and APX2 in NT plants and the increase was higher in this late isoform. Unexpectedly, a similar effect was observed in silenced *apx1/2* line, despite of the effectiveness of the hairpin to inhibit *apx1/2* transcription in control conditions (Fig. 2A and B). APX activity also was stimulated by high salinity in both genotypes but *apx1/2* roots displayed higher increase (150% in relation to control), whereas in NT plants the activity increased 40% compared to control condition (Fig. 2C). Despite the strong salt-induced increase in APX activity of silenced plants, the final reached activity in both treatments was lower than that presented by NT plants. Although drastic intensity of salt stress, the TBARS contents, an indicator of lipid peroxidation, did not change in both genotypes (Fig. 3A). It is important to note that the silencing triggered a prominent increase in H_2O_2 content, under control conditions (Fig. 3B). In opposition, the H_2O_2 content decreased in both NT and *apx1/2* plants, but more intensely (by 50%) in silenced roots, in response to high salinity. Nevertheless, this salt-induced reduction was enough to return to the initial H_2O_2 levels displayed by silenced roots to values around of those showed by NT plants under NaCl-treated condition (Fig. 3B).

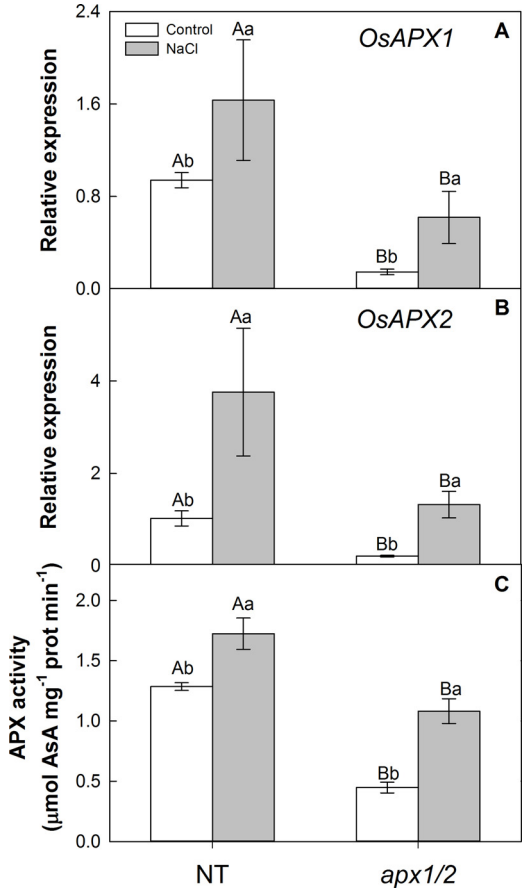

Fig. 2. Transcript levels of *OsAPX1* (A) and *OsAPX2* (B) and APX activity (C) in roots of NT and *apx1/2* plants exposed to NaCl (150 mM, $\Psi s = -0.62$ MPa) solution for eight days. Different capital letters represent significant differences among the NT and *apx1/2* plants within different treatments whereas different lowercase letters represent significant differences between treatments, within genotypes. Data are means of four replicates (±SD) and the averages were compared using the Tukey's test, at a confidence of 0.05. The relative expression of each APX locus was normalized by the average value obtained from the NT control plants.

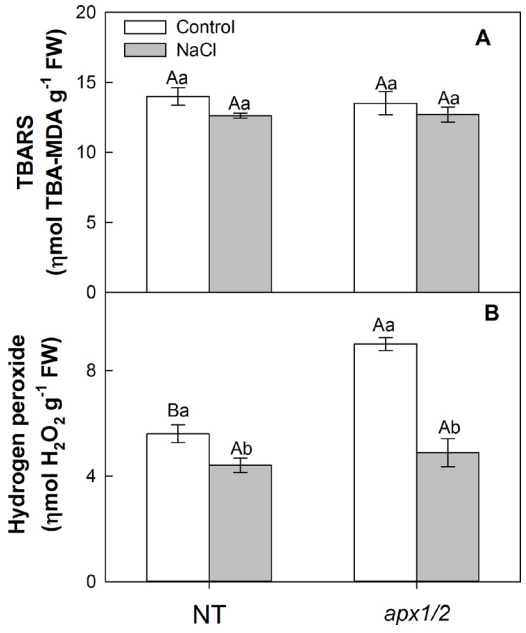

Fig. 3. Changes in TBARS (A) and hydrogen peroxide content (B) in roots of NT and *apx1/2* plants exposed to NaCl (150 mM, Ψs = −0.62 MPa) solution for eight days. Different capital letters represent significant differences among the NT and *apx1/2* plants within different treatments whereas different lowercase letters represent significant differences between treatments, within genotypes. Data are means of four replicates (±SD) and the averages were compared using the Tukey's test, at a confidence of 0.05.

3.4. High salinity strongly up-regulated others OsAPX and OsCAT transcripts but did not alter CAT activity in both genotypes

The APX1/2 silencing triggered slight changes in the expression of other *OsAPX* isoforms in roots, exception for *OsAPX8*, which was up-regulated by 5.6-fold compared to NT control. In opposition, high salinity induced strong up-regulation in important APX genes in NT plants such as the peroxisomal *OsAPX3* (60-fold), mitochondria *OsAPX5* (23-fold) and plastidial/chloroplastic *OsAPX8* (106-fold). Others (peroxisomal *OsAPX4* and mitochondria *OsAPX6*) were up-regulated in a minor extent, 3.8- and 4.42-fold, respectively. The effects of salt stress in silenced plants were minor: *OsAPX3* (5-fold), *OsAPX4* (5-fold), *OsAPX5* (4-fold) and *OsAPX8* (4.2-fold), all compared to NT control (Fig. 4). These results indicate that salt-induced up-expression of other APX transcripts, jointly to *OsAPX1* and *OsAPX2*, could had contributed intensely to increase APX activity in both root genotypes. Rice silencing triggered up-regulation in *OsCATA* and *OsCATB* mRNAs, which was strongly related to increase in CAT activity, compared to NT in control condition (Fig. 5). NaCl induced significant up-regulation in *OsCATA* and *OsCATB* transcript amounts, compared to NT control, but CAT activity did not change in roots of both genotypes. In compensation to CAT activity, POD and GPX activities were significant and similarly increased by salt stress and that increment was higher in POD activity (Table 1). Apparently, in response to increased salinity, the activities of APX, GPX and POD are more important to

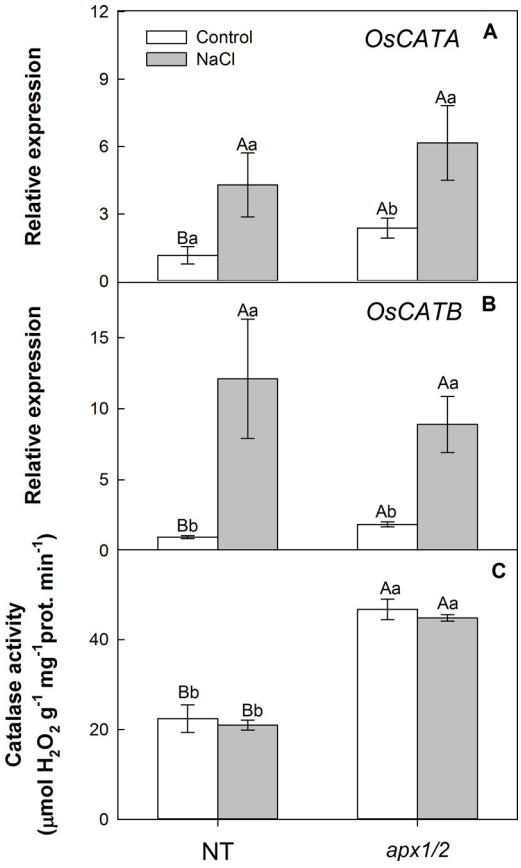

Fig. 5. Transcript levels of *OsCATA* (A) and *OsCATB* (B) and CAT activity (C) in roots of NT and *apx1/2* plants exposed to NaCl (150 mM, Ψs = −0.62 MPa) solution for eight days. Different capital letters represent significant differences among the NT and *apx1/2* plants within different treatments whereas different lowercase letters represent significant differences between treatments, within genotypes. Data are means of four replicates (±SD) and the averages were compared using the Tukey's test, at a confidence of 0.05. The relative expression of each APX locus was normalized by the average value obtained from the NT control plants.

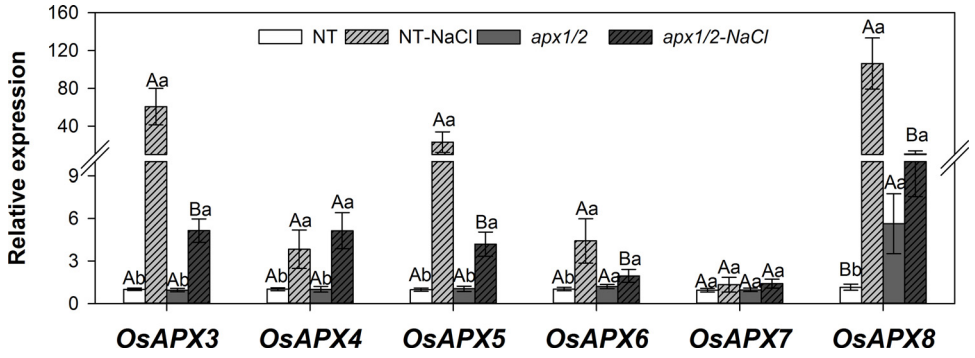

Fig. 4. Transcript levels of *OsAPX3-8* in roots of NT and *apx1/2* plants exposed to NaCl (150 mM, Ψs = −0.62 MPa) solution for eight days. Different capital letters represent significant differences among the NT and *apx1/2* plants within different treatments whereas different lowercase letters represent significant differences between treatments, within genotypes. Data are means of four replicates (±SD) and the averages were compared using the Tukey's test, at a confidence of 0.05. The relative expression of each *OsAPX* locus was normalized by the average value obtained from the NT control plants.

Table 1
Changes in POD activity (A) and GPX activity (B) in roots of NT and *apx1/2* plants exposed to NaCl (268 mM, Ψs = −0.62 MPa) or mannitol (Ψs = −0.62 MPa) solution for eight days. Different capital letters represent significant differences among the NT and *apx1/2* plants within different treatments whereas different lowercase letters represent significant differences between treatments, within genotypes. Data are means of four replicates and the averages were compared using the Tukey's test, at a confidence of 0.05.

Parameters	Rice lines					
	NT			*apx1/2*		
	Control	NaCl	Mannitol	Control	NaCl	Mannitol
POD activity[a]	7.6Bb	11.7Aa	7.6Bb	10.1Ab	12.6Aa	10.3Ab
GPX activity[b]	6.3Ab	12.5Aa	6.4Ab	5.7Aa	12.8Aa	4.9Bb

[a] μmol H_2O_2 mg^{-1} prot min^{-1}.
[b] ηmol NADPH mg^{-1} pro min^{-1}.

H_2O_2 scavenging and homeostasis maintaining than CAT, in both NT and silenced rice roots.

3.5. Mannitol did not cause change in root growth and lipid peroxidation associated to contrasting responses of CAT, POD and GPX activities in both genotypes

In contrast to NaCl, mannitol did not change the root FM in both NT and *apx1/2* genotypes (Table S1, Fig. S1E-F). Both *OsAPX1* and *OsAPX2* transcript amounts were up-regulated by mannitol in NT

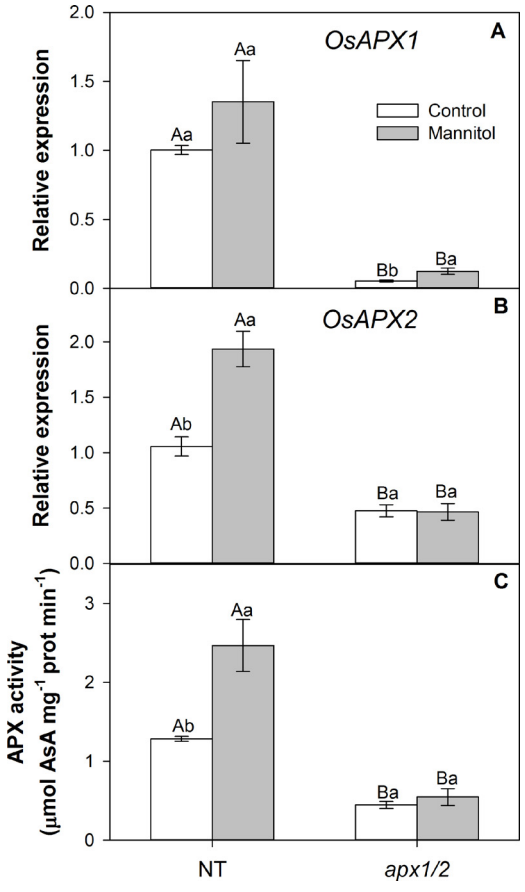

Fig. 6. Transcript levels of *OsAPX1* (A) and *OsAPX2* (B) and APX activity (C) in roots of NT and *apx1/2* plants exposed to mannitol (268 mM, Ψs = −0.62 MPa) solution for eight days. Different capital letters represent significant differences among the NT and *apx1/2* plants within different treatments whereas different lowercase letters represent significant differences between treatments, within genotypes. Data are means of four replicates (±SD) and the averages were compared using the Tukey's test, at a confidence of 0.05. The relative expression of each APX locus was normalized by the average value obtained from the NT control plants.

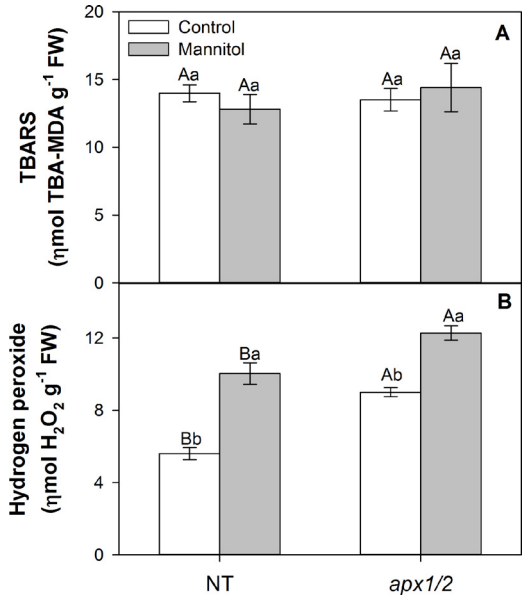

Fig. 7. Changes in TBARS (A) and hydrogen peroxide content (B) in roots of NT and *apx1/2* plants exposed to mannitol (268 mM, Ψs = −0.62 MPa) solution for eight days. Different capital letters represent significant differences among the NT and *apx1/2* plants within different treatments whereas different lowercase letters represent significant differences between treatments, within genotypes. Data are means of four replicates (±SD) and the averages were compared using the Tukey's test, at a confidence of 0.05.

but the late was much more over-regulated whereas *OsAPX1* and *OsAPX2* transcript levels remained very low and unchanged in *apx1/2* silenced roots in both control and mannitol conditions, compared to NT control (Fig. 6A and B). APX activity was strongly up-regulated by mannitol in NT and *OsAPX2* expression exhibited a similar response in both genotypes (Fig. 6C). Moreover, NT and *apx1/2* roots did not exhibit changes in TBARS levels in response to mannitol, similar to that previously noticed for salt stress (Fig. 7A). In opposition to the noticed for high salinity, mannitol induced a remarked increase in the H_2O_2 content in both genotypes (Fig. 7B).

Mannitol triggered up-regulation of some *OsAPX* transcripts in both genotypes, particularly *OsAPX3* and *OsAPX8*. The increase in the transcript amounts for NT and *apx1/2* were approximately and respectively: *OsAPX3* 2- and 5-fold; *OsAPX4* 2- and 2-fold and *OsAPX8* 2.5- and 12-fold (Fig. 8). It is interesting to note that the silencing triggered significant up-regulation (in control condition) in only *OsAPX8* (by 5.6-fold), all in comparison to NT control. NT-mannitol treated plants exhibited a strong up-regulation (by 12-fold) in *OsAPX8* compared with NT control. In addition, *OsCATA* transcripts were slightly up-regulated by mannitol in NT, whereas in *apx1/2* plants they significantly decreased. In contrast, *OsCATB* mRNA amounts increased in both genotypes, but the increment was higher in NT than in silenced plants (Fig. 9A and B). CAT activity strongly increased in both genotypes in response to mannitol, showing a trend very similar to *OsCATB* transcripts (Fig. 9C). In addition, differently from CAT, both POD and GPX activities did not change by effect of osmotic stress induced by mannitol and these responses were very contrasting to that displayed by these three enzymes in responses to high salinity (Table 1). In summary, high salinity and osmotic stress were able to differently trigger antioxidant mechanisms in rice roots. These responses allowed the plants to maintain adequate H_2O_2 levels and avoid oxidative stress, even when both cytosolic APXs were silenced (Fig. 10; Fig. S2).

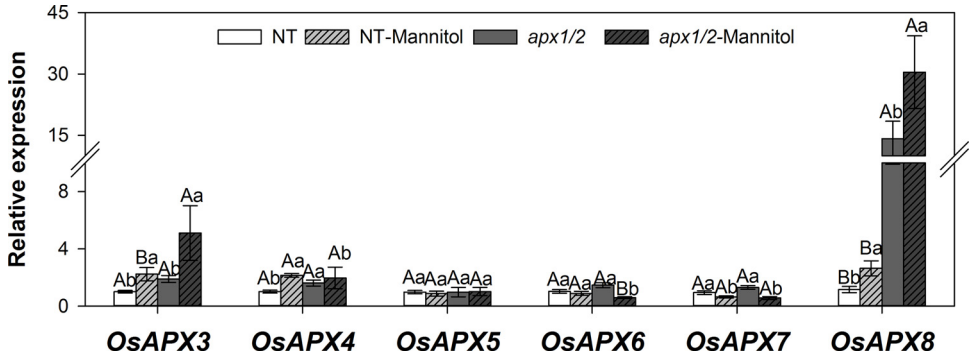

Fig. 8. Transcript levels of *OsAPX3-8* in roots of NT and *apx1/2* plants exposed to mannitol (268 mM, Ψs = −0.62 MPa) solution for eight days. Different capital letters represent significant differences among the NT and *apx1/2* plants within different treatments whereas different lowercase letters represent significant differences between treatments, within genotypes. Data are means of four replicates (±SD) and the averages were compared using the Tukey's test, at a confidence of 0.05. The relative expression of each *OsAPX* locus was normalized by the average value obtained from the NT control plants.

4. Discussion

The data reported here strongly evidenced that high salinity (NaCl) and mannitol triggered contrasting antioxidant response in rice roots, regardless of deficiency in cytosolic APXs. Interestingly, despite these differences, both stressful factors induced similar effects on membrane damage and lipid peroxidation in the two genotypes. These results clearly evidence that oxidative protection in rice roots against those stresses might involve redundant molecular and biochemical mechanisms as has been widely reported (Bonifacio et al., 2011; Miller et al., 2007; Rizhsky et al., 2002; Rosa et al., 2010; Carvalho et al., 2014; Sousa et al., 2015). Moreover, our data also show clearly that deficiency in both cytosolic APXs (APX1 and APX2) is less important or even insignificant for H_2O_2 scavenging and oxidative protection in cytosol of rice roots. These results are unexpected, once that is amply reported that these enzymes are essential to scavenging and homeostasis maintaining of H_2O_2 in leaves of some species (Davletova et al., 2005; Pnueli et al., 2003; Shigeoka and Maruta, 2014).

The presented data highlight that NaCl and mannitol should have triggered different signals to rice roots, which could have induced distinct perception, transduction and gene expression (Choi et al., 2014; Zhu, 2002). These genes could have encoded for differential expression of APX, CAT, POD and GPX enzymes (Foyer and Noctor, 2005), which might have acted in a redundant way, and differently for each genotype. It is interesting to note that the two stressful agents differently modulated the H_2O_2 levels and these responses were genotype-dependent. Thus, it is plausible to argue that this molecule can have acted as a signal, mediating the expression of genes linked to synthesis of those peroxidases (Corpas, 2015; Liu et al., 2010; Petrov and Van Breusegem, 2012; Sewelam et al., 2014). Indeed, a consensus has been established in literature that H_2O_2 is a powerful signaling molecule involved in expression of several defense genes against abiotic stress, particularly some peroxidases such as those studied here (Liu et al., 2010; Munné-bosch et al., 2013; Sewelam et al., 2014).

In this current study, H_2O_2 might have played a central role in antioxidant response to high salinity and osmotic stress in order to signal for different antioxidant response in rice roots. It is interesting to note that salt stress induced a prominent decrease in H_2O_2 content in silenced roots but did not change it in NT. In contrast, the content of this ROS was increased by mannitol in both genotypes. These responses were associated with differences in the expression of *OsAPX* and *OsCAT* isoforms and activities of the studied peroxidases in the two genotypes. These results suggest that H_2O_2 might have acted as a signaling for these different responses, which are stress and genotype dependent. Actually, in a previous study with cowpea roots, we have proposed that salt and drought stress conditions trigger distinct oxidative modulation, which involves activities of apoplastic class III peroxidases (POD) and APX (Maia et al., 2013). Indeed, under salt stress conditions, a strong decrease in APX activity and H_2O_2 concentration is inversely correlated to cell-wall POD activity.

Interestingly, in this current study both stressful factors, NaCl and mannitol, induced similar membrane damage, which was

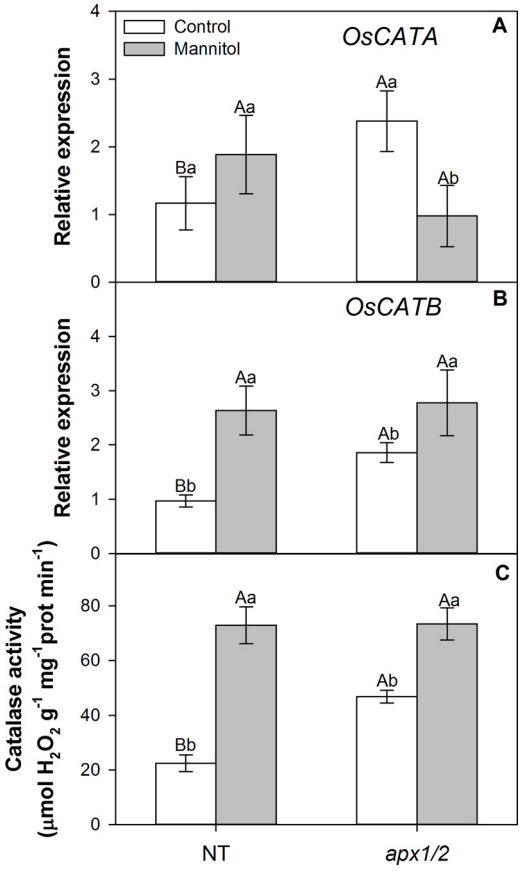

Fig. 9. Transcript levels of *OsCATA* (A) and *OsCATB* (B) and CAT activity (C) in roots of NT and *apx1/2* plants exposed to mannitol (268 mM, Ψs = −0.62 MPa) solution for eight days. Different capital letters represent significant differences among the NT and *apx1/2* plants within different treatments whereas different lowercase letters represent significant differences between treatments, within genotypes. Data are means of four replicates (±SD) and the averages were compared using the Tukey's test. The relative expression of each APX locus was normalized by the average value obtained from the NT control plants, at a confidence of 0.05.

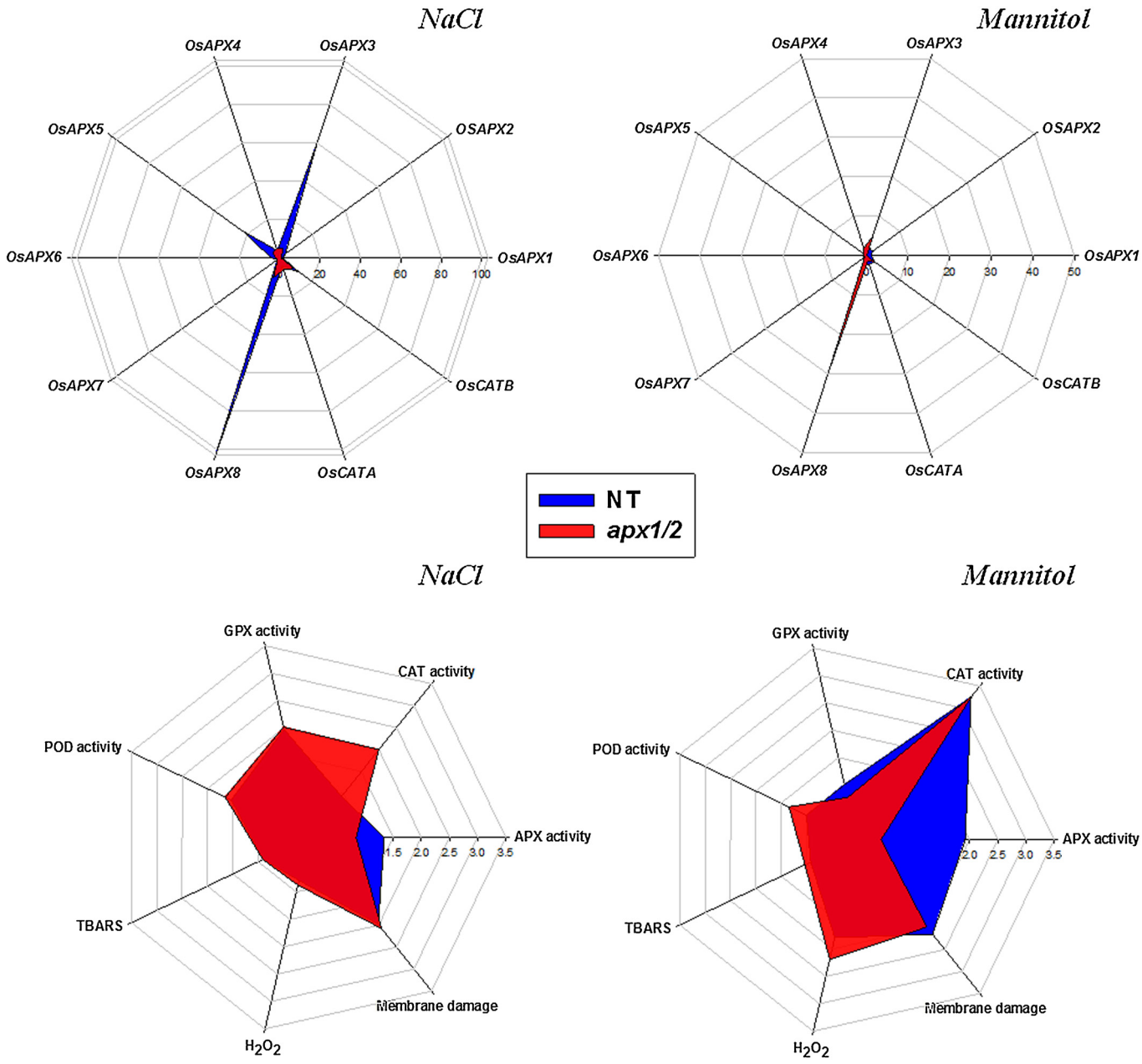

Fig. 10. Radar plot summarizing the different pathways affected in NT and *apx1/2* roots under salt and osmotic (mannitol) stress conditions. The graph illustrates *OsAPX* and *OsCAT* gene expression, oxidative stress parameters (TBARS, H_2O_2 content and Membrane damage) and enzymatic activities of important peroxidases (APX, CAT, GPX and POD activities). All the parameters were normalized by the respective value exhibited in NT rice roots under control conditions. Blue areas comprises the parameters modulated by NT roots. Red areas comprises the parameters modulated by *apx1/2* rice roots in comparison to NT. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

related to absence of oxidative stress, indicated by TBARS accumulation, in roots of both studied genotypes. These results are unexpected and they evidence a complex response. First, these results might suggest that both genotypes displayed efficient and similar protection against ROS accumulation in their roots, each one employing different antioxidant strategies. Second, as TBARS accumulation was not correlated to membrane damage, the obtained data could evidence that the dangerous effects caused on membrane integrity and/or permeability (electrolyte leakage) were not induced directly by ROS but possibly by NaCl and mannitol *per se*. Several reports have evidenced that plants display numerous antioxidant redundant (enzymatic and non-enzymatic) pathways to cope with excess ROS generated by abiotic stresses (Bonifacio et al., 2011; Miller et al., 2007; Rizhsky et al., 2002; Rosa et al., 2010; Sousa et al., 2015).

Some works have evidenced that in certain roots the TBARS accumulation is not frequently well correlated with cell integrity (measured by electrolyte leakage) – (Cavalcanti et al., 2007; de Azevedo Neto et al., 2006; Maia et al., 2013). The elucidation of the mechanisms involved with this apparent discrepancy is lacking. The excess H_2O_2 is the initiator for lipid peroxidation reactions via formation of hydroxyl radical by Fenton's reaction (Puppo and Halliwell, 1988). Therefore, the effective action of peroxidases under salinity and osmotic stress could minimize this process by means of H_2O_2 scavenging (Baxter et al., 2014; Passardi et al., 2004; Shigeoka and Maruta, 2014). GPX is especially important in this

process because its activity consumes organic hydroperoxide, blocking the lipid peroxidation chain reactions (Cavalcanti et al., 2007).

Interestingly, in this study each genotype and the two stressful factors induced the expression and activity of different peroxidases to cope with excess H_2O_2. These responses were probably related to toxicity avoidance and also to maintain different levels of this signaling molecule, enabling the triggering of distinct peroxidase gene expression (Baxter et al., 2014; Foyer and Noctor, 2015; Shigeoka and Maruta, 2014). Possibly, these responses are part of phenotypic plasticity, redox redundancy and compensatory mechanisms triggered by increased H_2O_2 in cytosolic APXs deficient rice roots (Baxter et al., 2014; Bonifacio et al., 2011; Miller et al., 2007; Mittler et al., 2004; Rosa et al., 2010).

Corroborating the above data, both high salinity and osmotic stress induced similar effects on root growth of the two studied genotypes, reinforcing that different physiological, biochemical and molecular mechanisms led to similar phenotypic characteristics. High salinity (150 mM NaCl) induced significant decrease in root fresh matter of both genotypes and this effect involved clearly osmotic and, possibly in a higher extent, ionic toxicity as revealed by intense Na^+ accumulation accompanied by strong reduction in K^+/Na^+ ratios. In opposition, mannitol did not change root biomass in both genotypes, evidencing a stress lesser dangerous than NaCl and corroborating that ionic component was very harmful to both genotypes. The data presented here reinforce that the high salinity components display distinct and complex responses in rice roots and that the deficiency (knockdown) of cytosolic APXs adds more complexity, as might be visualized in Fig. 10.

In conclusion, high salinity and osmotic stress induce contrasting antioxidant responses at level of change in expression of *OsAPX* and *OsCAT* isoforms in non-transformed and cytosolic APXs deficient rice roots. In addition, these two stressful factors also triggered very different modulation in activities of APX, CAT, GPX and POD. All these responses were associated with changes in the H_2O_2 levels, suggesting this signaling molecule as a central player. As both stresses induced similar effects in NT and cytosolic APXs deficient roots, in terms of lipid peroxidation, cell integrity and root matter accumulation, it is plausible to conclude that cytosolic APXs deficiency is dispensable to oxidative protection in roots. It is also evident that rice roots deficient in these enzymes are able to display phenotypic plasticity to cope with high salinity and osmotic stress.

Conflicts of interest

The authors have no conflicts of interest to declare.

Contributions

JRC conducted all experiments, performed biochemical determinations, and participated of the interpretation and discussion of results. MCLN performed biochemical determinations. FELC interpreted the results and participated of the manuscript writing. MOM performed APX1 and APX2 westernblot analyze and conducted some experiments. DJM performed determinations of qRT-PCR. MMP designed and obtained the transformed plants, supervised the determinations and interpreted the results of qRT-PCR. JAGS was the mastermind of the research and wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.envexpbot.2016.07.002.

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