Contrasting responses of plastid terminal oxidase activity under salt stress in two C4 species with different salt tolerance

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Abstract

The study reports the responses of photosynthesis to NaCl stress in two C4 species: a glycophyte Setaria viridis (SV) and a halophyte Spartina alterniflora (SA). SV was unable to survive following exposure to NaCl level higher than 100 mM, in contrast, SA could tolerate NaCl up to 550 mM. Under different O2 concentrations, SV showed an increased P700 oxidation level following NaCl treatment, while SA showed almost no change. We also observed an activation of the NDH-dependent cyclic pathway in SV by about 2.4 times upon exposure to 50 mM NaCl for 12 days; however, its activity in SA dropped by about 25%. Using PTOX inhibitor (n-PG) and inhibitor of the Qo-binding site of Cytb6/f (DBMIB) to restrict electrons flow towards PSI, at either 2% or 21% O2, we showed an enhanced plastid terminal oxidase (PTOX) activity for SA but not for SV under NaCl stress. We further showed that both the mRNA and protein levels of PTOX increased by about 3˜4 times for SA under NaCl stress but not or much less for SV. All these suggest that the up-regulation of PTOX is a major mechanism used by halotype C4 species SA to cope with salt stress.

1 INTRODUCTION

Soil salinity is a major environmental stress that adversely affects crop productivity and harvest quality (Horie & Schroeder, 2004). Approximately one fifth of the world's cultivated area and about half of the world's irrigated lands are affected by salinization (Sairam & Tyagi, 2004). Mechanisms of how plants respond and/or tolerate salt stress are under intensive study (Zhu 2001; Munns & Tester, 2008). To survive and overcome salt stress, plants respond and adapt with complex mechanisms that include developmental, morphological, physiological and biochemical strategies (Taji et al., 2004; Acosta-Motos et al., 2015), which serve to modulate ion homeostasis, osmolyte biosynthesis, compartmentation of toxic ions, and reactive oxygen species (ROS) scavenging systems (Stepien & Klobus, 2005; Flowers & Colmer, 2008; Stepien & Johnson, 2009). In this study, we report that a protein involved in alternative electron transfer, PTOX, might be related to salt tolerance in C_4 plants.

The protein PTOX is a plastid-localized plastoquinol oxygen oxido-reductase that was discovered in the so-called immutans of*Arabidopsis thaliana* which shows a variegated leaf phenotype (Rédei, 1963; Wetzel et al., 1994, Carol et al., 1999; Wu et al., 1999; Shahbazi et al., 2007). In chloroplasts, PTOX is located at the stroma lamellae facing the stroma (Lennon et al., 2003) and it is essential for the plastid development and carotenoid biosynthesis in plants (Carol et al., 1999; Aluru et al., 2001). PTOX is also involved in photosynthetic electron transport (Okegawa et al., 2010; Trouillard et al., 2012), chlororespiration (Cournac et al., 2000), poising chloroplast redox potential under dark (Nawrocki et al., 2015), and in stress response (McDonald et al., 2011; Sun and Wen, 2011). Plants grown in moderate light under non-stress conditions have low PTOX concentrations (about 1 PTOX protein per 100 PSII; Lennon et al., 2003); in contrast, elevated PTOX levels have been reported in plants exposed to abiotic stresses such as high temperatures,

high light and drought (Quiles, 2006), high salinity (Stepien & Johnson, 2009), low temperatures and high intensities of visible light (Ivanov et al., 2012) and UV light (Laureau et al., 2013).

In this study, in an effort to understand potential mechanism of how the halophyte *SA* tolerate high salt stress, we show that compared to a glycophyte species *SV* , under high salt stress (500 mM),*SA* showed increased expression of PTOX, which might have played a critical role for the maintenance of photosynthetic physiology and hence high photosynthetic efficiency of this species under salt stress.

2 MATERIALS AND METHODS

2.1 Plant Material

Seeds of *Spartina alterniflora* (*SA*) were collected from*San-San* Lake in South East Shanghai city at the end of November in 2015 and 2016. The cleaned spikelets were stored in wet tissue (cloth) in sealed plastic at 4°C in the refrigerator. *SA* mature seeds require two to three months, after-ripening, wet storage in cold (stratification) to break dormancy (Garbisch & McIninch, 1992) and they remain viable for about one year. Seeds of *SV* were rinsed several times with tap water and then transferred to Petri-dishes and covered with water till germinate. After germination, they were transferred into potted soil. When the young seedlings of *SA* were about 2 cm in length and started greening, they were removed from the glass petri dishes. Trays containing *SA* seedlings were kept indoor at a temperature between 25˜27°C, under fluorescent light at a PPFD of 80 - 120 μmol m^{-2} s^{-1} with a photoperiod of 16/8 hour (light/dark). Two-month old healthy plants with large expanded leaves where transferred to a hydroponic system for salt treatment. For *SV* , dry seeds were directly sown into wet potted soil, which was maintained wet by spraying water daily on the soil till the seeds germinated. *SV* grew under the same photoperiod and temperature conditions as *SA* . Nutrient was added routinely to ensure healthy growing plants before transferring them to hydroponic medium for salt stress treatment.

2.2 Salt stress (NaCl) treatment

Salt (NaCl) treatment was applied to hydroponic solution. During the plants transfer, roots were washed adequately with tap water then rinsed with deionized water. Four-week-old *SV* and 10-week-old *SA*plants were treated with 0, 50, 100, 250, 400, and 550 mM NaCl for up to 15 days. The composition of the hydroponic medium was as described by Hoagland and Arnon (1950).

2.3 Determination of monovalent cations ($Na^+$and K^+) content

Leaves were harvested and washed with deionized water. The leaf samples were dried first at 105°C for 2 h, subsequently at 70°C for 72 h, and then weighted for dry weight. Lyophilized leaves were milled to powder for mineral nutrient analyses. Powdered samples were extracted with 10 ml of HNO_3 (0.1N) for 60 min at 95 ºC. The resulting solutions were filtered through Whatman filter paper, diluted and analyzed for Na^+ and K^+. Cation concentrations were determined with an atomic absorption spectrophotometer (PerkinElmer, PE AAS 900 F).

2.4 Chlorophyll (Chl) content measurements

Chl content was determined according to Porra et al. (1989). Leaf segments (0.1 g) were first washed with distilled water and then kept in 1 ml acetone (80%) at 4°C for one to two weeks. Then samples were centrifuged at 13,000 g for 5 min and subsequently their absorbance was monitored at 663 and 645 nm using a UV visible spectrophotometer (50 Bio Varian, Varian Inc., Walnut Creek, CA). Total Chl content was calculated according to the following equation: total Chl ($mg{\cdot}L^{-1}$) = (8.02 x OD_{663}) + (20.21 x OD_{645}), where OD stands for optical density. The results of the Chl content were expressed as mg per gram fresh weight (mg g^{-1} fresh weight (FW)) and calculated based on the extinction coefficients and the equations given by Porra et al. (1989).

2.5 Assessment of photosystem II parameters

PSII efficiency was assessed using the Chl *a* fluorescence induction (FI) technique. We used the multifunctional plant efficiency analyser (M-PEA; Hansatech, King Lynn, Norfolk, UK) for the evaluation of PSII parameters as described in details by Essemine et al. (2017). Plants were dark-adapted for at least one hour at 25degC before measurements. Then, healthy and fully expanded leaves were exposed to saturating orange-red (625 nm) actinic light (5,000 μmol m^{-2} s^{-1}) provided by the LED for 1 second. The ratio of variable fluorescence level $F_v(F_m\text{-}F_0)$ to maximal fluorescence level F_m (F_v/F_m) was used to evaluate the maximum efficiency of PSII. F_m (P-level) represents the maximum yield of Chl *a* fluorescence and F_0 (O-level) is the minimum Chl *a* fluorescence (the intensity of Chl *a* fluorescence of dark-adapted sample with a measuring beam of negligible actinic light intensity). F_v/F_0 parameter represents the functional reaction center of PSII. All the parameters listed in the Table S1 were calculated from the original OJIP curves according to the so-called JIP-test (Strasser et al., 2004)

2.6 Setting of PAM together with Infrared gas analyzer to control CO_2 and O_2 supply

A special chamber was custom-designed and developed to enable precise control of CO_2 and O_2 environments. This chamber was tightly mounted on the detector-emitter of the Dual-PAM-100 fluorimeter which was connected through a hole to the Li-COR 6400 portable infrared gas analyzer to control CO_2 supplies (390 or 2000 μL L^{-1}) by Li-cor and via another window to an oxygen source equipped with an oxymeter to adjust the flow of oxygen from the source to the chamber. Oxygen sources with different concentrations (e.g. 2 and 21% as used in this study) are supplied by a gases distribution station (GDS). The setting for experiments using different levels of CO_2 and O_2 was as depicted in Figure 1 and video in supplemental data.

2.7 Evaluation of P_{700} redox state in leaves of SV and SA

In order to monitor the photosynthetic electron flow through PSI during steady state photosynthesis *in vivo* , we estimated the redox state of P_{700} in the light by measuring oxidation of P_{700} within the leaf as absorbance changes at 830 minus 875 nm. P_{700} was oxidized to $P_{700}{}^{+}$ at different intensities of actinic light ranging from 0 to 1804 μmol $m^{-2}s^{-1}$ (ΔA) then reduced in the dark and finally oxidized to a maximum level of $P_{700}{}^{+}$ under far-red illumination to favor PSI photochemistry (ΔAmax; Klughammer and Schreiber, 1994; Zygadlo et al., 2005; Klughammer & Schreiber, 2008). The light dependence of the P_{700} oxidation ratio (ΔA/ΔAmax; Klughammer & Schreiber, 1994; Zygadlo et al., 2005; Klughammer & Schreiber, 2008; Dalcorso et al., 2008) was examined in*SV* and *SA* plants. The Far-red light (FR) intensity used was 102 μmol m^{-2} s^{-1} and a 100 ms saturation pulse (SP) of PPFD of 8000 μmol $m^{-2}s^{-1}$ was applied under background AL and FR.

2.8 Conductance of the electron transport chain (g_{ETC})

To estimate the conductance of the electron transport chain*(g_{ETC})* , we used a similar experiment setting to the previous section monitoring the redox state of PSI with slight modifications. The saturating pulse was given under darkess simultaneously with the termination of AL. Notably, a 100-ms width SP at a PPFD of 8000 μmol $m^{-2}s^{-1}$ was applied and the decay in absorbance followed upon transition from the 100 ms SP to darkness (Klughammer & Schreiber, 1994; Klughammer & Schreiber, 2008). This intensity was found to be saturating across all conditions used. Under these conditions, application of a flash induced a rapid rise in the absorbance signal, with no decrease during the duration of the flash (not shown). The absorbance decay curve under such conditions (ctrl or salt) approximated closely to a first-order reaction and was fitted well with a mono-exponential curve, yielding a rate constant. This was taken as a measure of the conductance of the electron transport chain (Golding & Johnson, 2003; Stepien & Johnson, 2009).

2.9 In situ histochemical localization of reactive oxygen species (ROS)

To detect reactive oxygen species (ROS), histochemical staining with nitroblue tetrazolium (NBT) was performed following Dong et al. (2009) with minor modifications. Detached leaves were first vacuum-infiltrated in their appropriate solution (with or without NBT). For superoxide free radical ($O^{-}{}_2{}^{\cdot}$) characterization, leaf samples were soaked in 6 mM NBT solution containing 50 mM sodium phosphate (pH 7.5) for 12 h under darkness. To detect hydrogen peroxide (H_2O_2), detached leaves were immersed in 5 mM of 3, 3' diaminobenzidine (DAB) solution containing 10 mM MES (pH 3.8) for 12 h under darkness. After that, the

adaxial surface of the leaf was exposed to moderately high light (500 μmol m^{-2} s^{-1}) for 1h. The dark-blue spots reveal the interaction between NBT and the generated $O^{-}{}_2{}^{\cdot}$; however, the brown spots on the leaf reflect the interaction between DAB and formed hydrogen peroxide (H_2O_2) at the presence of peroxidase. Both reactions (DAB and NBT) were stopped by soaking the leaves with lacto-glycerol-ethanol (1:1:4 by vol). Chl was removed from the leaves prior to imaging by boiling leaves in their respective solutions (NBT or DAB) for 2 min then the solutions were discarded these solutions and leaves were re-boiled in water for 2 to 3 times (1 min each). Then leaves were incubated in alcohol (99.5%) as described by Zulfugarov et al. (2014) till complete removal of Chl. Afterwards, leaves devoid of Chl were preserved in 50% ethanol till photographed.

2.10 RNA extraction, purification and qRT-PCR analysis

Eight candidate housekeeping genes (Kumar et al., 2013) were screened to select an appropriate reference gene for *SA* and *SV*. These eight genes have been reported on *Setaria italica* (Foxtail Millet), representing different functional classes and gene families (Kumar et al., 2013). These genes are: viz., 18S rRNA (18S), elongation factor-1a (EF-1a), actin2 (Act2), alpha tubulin (Tub α), beta tubulin (Tub β), translation factor (TLF), RNA polymerase II (RNA POL II), adenine phosphoribosyl transferase (APRT; Kumar et al., 2013). In a recent study on *Spartina alterniflora* , tubulin was used as a housekeeping gene (Karan and Subudhi, 2012a). Based on the similarity index between the sequences of each housekeeping gene in *SV* and*SA* , we obtained the highest similarity index in Tubulin alpha (Tub α), which is around 85%. In this study, we therefore selected Tub ? as reference gene for qRT-PCR.

Total RNA was extracted from mature leaves using Purelink RNA Mini Kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Concentration of each RNA sample was measured using NanoDrop 2000 spectrophotometer (NanoDrop Technologies). Leaves were sampled from both species and total RNA was extracted using TRIzol Plus RNA Purification kit (Invitrogen Life Technologies, http:// www.invitrogen.com). One microgram (1 μg) of total RNA was used to synthesize first strand cDNA with SuperScript VILO cDNA Synthesis Kit (Invitrogen Life Technologies, http://www. invitrogen.com). Quantitative real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, USA) with the fist strand cDNA as a template on a Real-Time PCR System (ABI StepOnePlus, Applied Biosystems lco., USA), with the following cycling parameters: 95°C for 10 s, 55°C for 20 s, and 72°C for 20 s. Primers for qRT-PCR were designed using Primer-Blast of the National Center for Biotechnology Information website (NCBI; https://www.ncbi.nlm.nih.gov). The primers for PTOX and Tubulin-alpha used for qPCR analysis were listed in Table S2. Relative expression of gene against housekeeping gene tubulin-alpha was calculated as: 2^{-CT} (ΔCT = CT, gene of interest-CT, Tubulin-alpha), as described by Livak & Schmittgen (2001). Six complete biological and technical replicates were determined for the analysis.

2.11 Detection of PTOX contribution in electron transport in SA

To determine the contribution of the PTOX to overall PSII electron transport, the leaves of control and salt-treated *SV* and*SA* were vacuum infiltrated with either water or with 5 mM*n* -propyl gallate (n-PG, 3,4,5-trihydroxy-benzoic acid-n-propyl ester; Sigma) or 50 μM DBMIB (2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone, Sigma). The stock solutions of *n* -PG were prepared in ethanol and DBMIB in methanol. All inhibitor solutions were prepared fresh.

2.12 Western-blot analysis

For immunoblot (western-blot) analysis, thylakoids were isolated as described in Cerovic & Plesnicar (1984). Thylakoids proteins were extracted from membranes using 125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 0.1% (w/v) bromophenol blue. Protein concentration was estimated using a Bio-Rad protein assay kit (Bio-Rad Laboratories). Immunoblotting was performed as described in Mudd et al. (2008). Polyclonal antibodies raised against PTOX for both species (*SA* and *SV*) were designed by the company according the sequence homology between species which was 63% (see blast sequences alignment results in supplemental data).

3 RESULTS

3.1 Chlorophyll a fluorescence induction and JIP test

We used a JIP-test (Strasser et al., 2004) to unravel the effect of salt stress on most of PSII parameters in both *SV* and *SA* . Results depicted in Figure 2 were derived from the fast phase Chl*a* fluorescence induction curve, i.e. the OJIP curve. Salt stress treatment experiments show that, for *SV* , even moderate NaCl concentration (50 mM) increased the Fo level (data not shown) and the J-test of OJIP induction curves (data not shown). However, *SA*showed no/or slight difference in the OJIP induction curves for moderate (250 mM) and high (550 mM) NaCl concentrations compared to the control without NaCl. Therefore, the function of PSII was not affected for*SA* ; however, it was strongly inhibited and/or damaged for*SV* even at relatively low NaCl concentration (50 mM). To study in detail the effects of NaCl on PSII in these two species, we evaluated the PSII parameters using the JIP-test (Fig 1, Table S1; Strasser et al., 2004). The JIP-test was evaluated from *SV* (A, B and C) and*SA* (D, E and F) exposed for 5 (A, D), 10 (B, E) and 15 days (C, F) to different NaCl concentrations. Herein, we observe that after 5 days of exposure to 100 mM NaCl, PSII parameters showed apparent change in *SV* (Figure 2A, green spider). The salt effect became more pronounced after 10 days of exposure to salt at either 50 or 100 mM (Figure 2B, red and green spiders). However, for *SA* , the deviation in the PSII parameters calculated with JIP-test was much less and observable only for high NaCl concentration (550 mM) after 10 and 15 days' exposure (Figure 2E-F, black spider). Therefore, PSII of *SV*was more sensitive to salt stress, as compared to *SA* .

3.2 Sodium and potassium sequestration in leaf following NaCl treatments

Plants of *SV* and *SA* were grown for 4 or 8 weeks before their exposure to a range of salt concentrations. Exposure of *SV*to NaCl concentrations higher than resulted in plant death before the end of the experiment; so higher NaCl concentration treatments were not used for *SV* . Exposure of *SA* to NaCl concentration up to did not result in considerable mortality. The concentration of Na^+ in control leaf tissue was considerably higher in*SA* than in *SV* (Figure 3A-B). This difference disappeared after exposure to salt, due to a quick accumulation of Na^+ in the leaf of *SV* . The accumulation of Na^+ in leaves of *SA* was much lower at external NaCl concentrations between 0 and . Na^+ accumulation increased sharply in *SV* leaves over the experiment (Figure 3A), whereas leaf Na^+ content in *SA* increased less, even at higher external concentrations of NaCl (Figure). The Na^+ levels measured after 12 days NaCl treatment in Spartina exposed to 400 and NaCl was nearly similar to that of *SV*subjected to only NaCl (Figure 3A-B). At NaCl, *SV* accumulated more NaCl in the leaf than *SA* under all salt concentration range (100-550 mM). This is owing to the exclusion of NaCl to the leaf surface for Spartina. This exclusion mechanism represents a second barrier of*SA* defense against high NaCl concentrations besides the sequestration of salt in the vacuole. Earlier study performed on halophyte *Aeluropus littoralis* , a species that can tolerate up to NaCl, showed that an increase in leaf epidermal thickness was mainly due to an increase in cell size following salt accumulation (Barhoumi et al., 2007).

SA and *SV* differed also in their K^+concentrations in the leaf. Herein, the concentration of K^+ in leaf tissue of plants watered with salt-free medium was higher by about 30% in *SA* leaves (Figure 3C-D). Following salt treatment, the K^+ content of the leaf in *SV* decreased considerably, especially after 4 and 8 days treatment at 100 mM NaCl. However, in *SA* , there was an initial increase in K^+ with the increase in NaCl concentration; after 12 days of treatments, the K^+concentration in the leaf gradually decreased with an increase in the NaCl concentration (Figure 3D). This also reflected by the ratio k^+/Na^+ (Figure 3E-F), where we observed a dramatic decline in this ratio for *SV* but very less and mostly maintain stable with time course in *SA* , especially at NaCl concentrations higher than 250 mM (Figure 3E-F).

3.3 Chl content in leaf and non-photochemical quenching decay components: $NPQ_{fast,\ slow}$

The total Chl content in untreated *SA* leaves under salt treatment was around 4.5 times higher than that in untreated *SV* leaves (Figure 4A-B). Exposure of *SV* to NaCl resulted in a progressive decrease in Chl content (Figure 4A); the total Chl concentration after 12 days of salt treatment with 50 and 100 NaCl dropped by 42% and 58%, respectively. In contrast, treatment of *SA* with 50 and 100 NaCl did not result

in any significant decline in the total Chl content except at NaCl concentrations higher than , e.g. at NaCl treatment, there as a ˜20% decrease in total Chl content (Figure 4B).

In *SV* , NaCl treatment resulted in an increase of NPQ, while NPQ remained similar or oightly increased in *SA* at all NaCl concentrations (Figure 4C-D). The NPQ increase in *SV* might be resulted from a modulation of either a protective high-energy-state quenching or otoinhibition, which differ in the relaxation kinetics after actinic light illuminationell & Johnson, 2000; Johnson et al., 2009). MeasurementNPQ were taken after 16 daosure to 100 and 400 mM NaCl treatments for *SV* and *SA* , respectively (Figure 4C-D). The NPQ recovery under darmeasured to quantify the magnitude of each phase of NPQ dark decay. In *SV* , quantification of the fast and slow relaxing components of NPQ quenching showed that the majority of quenching relaxed rapidly in the dark (NPQf), indicating that it was high-energy-state quenching (Figure 4C-D); while a part of the quencas more conservative (NPQs), suggesting the occurrence of photoinhibition in leaves of *SV* due to high NaCl. Both forms of NPQ quenching increased in response to salt treatment (Figure 4C-D). The increase in total NPQ in *SA* was comparatively less and was mainly due to an increase in NPQ_f (photoprotection process).

3.4 Electrons flow to molecular oxygen under salt in both C_4 species

The electron generated by H_2O splitting can be used by alternative sinks, in addition to the common sink to support NADPH generation. The most commonly known sinks are the reactions involving oxygen, including photorespiration and Mehler reaction (Chen et al., 2004; Shirao et al., 2013). To assess the relevance of these pathways, the oxygen dependence of electron flow was performed. *SV* and*SA* were subjected to different Actinic Light at a range of irradiance levels (AL, 0 to 1806 µmol m-2 s-1) at the presence of saturating CO_2 (2000 µL L^{-1}) and either 21% or 2% of O_2. Regardless the degree of NaCl treatments, the ERT_{II} in both species reached its maximum at around 400 µmol m^{-2} s^{-1}(Figure 5A-B). Exposure of control plants to low oxygen (2%) resulted in a decrease of PSII ETR at saturating irradiances.

Measurements of the redox state of the primary electron donor of PSI (P_{700}) revealed slight effects of oxygen concentration in control plants. With increasing irradiance, P_{700}became progressively more oxidized in both species (Figure 5C-D). Despite the proportion of oxidized $P_{700}(P_{700}{}^{+})$ was insensitive to the oxygen concentration in control untreated plants, the conductance of the ETC (g_{ETC}; Figure 5E-F) declined and the ETRI follows the same trend and decreased by the same amount (Figure 5G-H).

SV exposed to 100 mM NaCl showed lower ETR_{II} at high CO_2 than the control plants (Figure 5A). As in the control, electron transport through PSII decreased slightly under low O_2 (Figure 5A). In contrast, exposure to NaCl resulted in an increase in the ETR_{II} in *SA* compared to untreated plants; this increase in electron transfer through PSII was completely revoked under low O_2 concentration (Figure 5B).

The proportion of oxidized $P_{700}(P_{700}{}^{+})$ in salt-treated *SV* was significantly higher under low O_2 (Figure 5C). This is accompanied by to a negligible decline in the *g* ETC, resulted in a slight increase in the electron flow through PSI potentially via cyclization across FRQ (Figure 5G). Conversely, in *SA* , PSI ETR (ETR_I) in the absence or presence of salt (250 mM) decreased at low O_2 (Figure 5H). This caused by an enhancement in P_{700} oxidation and a fall in g_{ETC} (Figure 5D-F).

3.5 Activity of NAD(P)H dehydrogenase (NDH)-dependent cyclic electron flow in both species under salt stress

NDH cyclic pathway activity around PSI was assessed as the post-illumination rise (PIR) of F_o Chl fluorescence was monitored after switching off actinic light Essemine et al. (2016). The magnitudes of PIR for *SV* and *SA* under both control and salt stress conditions were displayed in Figure 6. Under normal conditions, we observe more than two times higher NDH activity in*SA* than in *SV* (Figure 6). The results show as well an increase in the NDH in leaves of *SV* plants endured 50 mM NaCl for 12 days by about 2.36 times (Figure 6). However, *SA* plants exposed for the same time period (12 days) to 250 mM NaCl exhibited a significant decrease (about 25%) in the NDH activity (Figure 6). This is very likely attributable to the activation of PTOX in Spartina under salt stress. Hence, the activity of PIR declines in *SA* in favour of that of PTOX.

This reflects the existence of an efficient competition between these two pathways (PTOX and NDH) for the oxidation/reduction of the PQ pool, respectively. Eventually, the oxidation of the PQ pool by PTOX overcomes its re-reduction by NDH cyclic (Figs. 6 and 8). So far, PTOX may represent an alternative pathway to cyclic and linear routes for the protection of *SA* against intersystem over-reduction and minimize or avoid damages to both photosystems (PSI and PSII). Thereby, it may function as a safety valve for the photosynthetic transport chain. In this regard, our findings are in line with that of Ahmad et al. (2012), where authors have shown a decrease in the PIR in tobacco overexpressing PTOX from *Chlamydomonas reinhardtii* (Cr-PTOX) compared to WT (Ahmad et al., 2012) and they demonstrated that the decrease in PIR is attributed to the enhanced activity of PQ pool oxidation by the high level of PTOX protein in the over-expressed line.

3.6 Plastid terminal oxidase (PTOX) as a plastohydroquinone:oxygenoxidoreductase

The improved efficiency and/or the additional turnover of PSII under salt treatment in spartina at the presence of 21% oxygen, compared to either control with 21% O_2 or 250 mM NaCl with 2% O_2, is very likely attributed to electron transfer directly to molecular oxygen (O_2). Since experiments were conducted under a saturating CO_2 concentration of 2000 $\mu L\ L^{-1}$, we exclude the contribution of photorespiration to this effect. Usually, the photo-reduction of O_2 may occur at the acceptor side of PSI via the Mehler reaction; however, the lack of a sensitivity of PSI parameters to oxygen suggests that this is unlikely the reason, or at least not the only reason. So here we test the possibility that the putative quinone-oxygen oxidoreductase, the plastid terminal oxidase (PTOX) or IMMUTANS protein (Shahbazi et al., 2007; Heyno et al., 2009) might have played a role as well for *SA* .

To determine whether the PTOX may play a role in electron transfer from PSII to O_2, measurements of ETR_{II} were performed on leaves obtained from control and salt-treated *SA* and*SV* which were vacuum infiltrated with either water or a solution of the PTOX inhibitor *n* -propyl gallate (*n* -PG; 3,4,5-trihydroxy-benzoic acid-*n* -propyl ester; Joët et al., 2002; Josse et al., 2003; Kuntz, 2004; Rosso et al., 2006; Houille-Vernes et al., 2011; Sun & Wen, 2011; Trouillard et al., 2012; Shirao et al., 2013; Nawrocki et al., 2015). In *SV* , PSII quantum yield (F_{PSII}) was insensitive to *n* -PG, regardless whether the plants have been exposed to NaCl treatment or not (Figure 7A). This was also the case for control *SA* . In *SA* exposed to 250 mM NaCl, F_{PSII} was insensitive to *n* -PG (Figure 7B). F_{PSII} measured 12 days after initiating NaCl treatment was reduced by about 32 and 45%, in leaves infiltrated with 5 mM *n* -PG, in the presence of 21 and 2% O_2, respectively (Figure 7B), falling thereby to the control level or even slightly lower (Figure 7B). Interestingly, at low O_2 in salt-stressed plants, we observed a decrease in the F_{PSII}. This suggests strongly that molecular oxygen (O_2) may act as a terminal electron acceptor by oxidizing the plastoquinol (PQH2).

The effect of *n* -PG suggests a potential activity of plastid terminal oxidase (PTOX) located on the stromal side of the membrane in*SA* though this does not exclude a potential contribution of the Mehler reaction to electron transport. To measure electron flow to oxygen excluding any contribution of the Mehler reaction, leaves were infiltrated with the cytochrome b_6/f ($Cytb_6/f$) inhibitor dibromothymoquinone or 2,5-dibromo-3-methyl-6-isopropylbenzoquinone (DBMIB), a specific inhibitor of the Q_o-binding site (Malkin, 1981, 1982; Rich et al., 1991; Schoepp et al., 1999). In *SV* , this almost completely abolished the F_{PSII} and thereby the electron flow beyond the $cytb_6/f$, regardless the NaCl treatment (Figure 7C). In control *SA* leaves, DBMIB also strongly inhibited F_{PSII}, though a residual F_{PSII} and also electron transfer remained. In salt-stressed *SA* leaves, DBMIB only partially inhibited F_{PSII} with decreasing O_2 concentration resulting in greater inhibition of F_{PSII}. The extent of DBMIB insensitive, oxygen-sensitive F_{PSII} decrease was similar to that of*n* -PG-sensitive electron transport in the same leaves (Figure 7B and D).

The dramatic decline in F_{PSII} in the presence of DBMIB at low O_2 in salt treated Spartina leaves (Figure 7D) might be explained as a double restriction in the electrons flux beyond PSII. First limitation due to the blockage (or shortage) in the electrons flow towards PSI due to the presence of DBMIB and the second curtailment is tightly linked to the drop in the O_2level (2%).

Western-blot analyses of thylakoid membrane extracts of *SA* and*SV* using antibodies raised against *Zea*

mays PTOX revealed the presence of a 35-kDa band in both species (Figure 8). For untreated plants, *SA* showed higher protein abundance than in *SV* . In the latter (*SV*), salt treatments resulted in a slight increase in the PTOX abundance (Figure 8A and inset), though the expression level of PTOX transcript insignificantly decreased (Figure 8B). In *SA* , treatment with 250 mM NaCl elevated PTOX abundance by 3˜4 times compared to the control (Figure 8A and inset). Similarly, the transcript abundance of PTOX was also elevated under NaCl treatment by the same amount (Figure 8A-B).

3.7 Reactive oxygen species generation under salt in C_4 species

Histochemical staining with nitroblue tetrazolium (NBT) shows the appearance of dark-blue spots on the edge of *SA* leaves exposed to 250 mM NaCl for 12 days (Figure 9B). This dark-blue staining reveals the interaction between NBT and the generated superoxide free radical ($O^{-}_{2}\cdot$) following exposure to moderately high light (500 µmol m^{-2} s^{-1}). However, these dark-blue spots were spread all over the surface of *SV* leaves subjected to 50 mM salt for 12 days (Figure 9D), suggesting that salt treatment dramatically increased the production of reactive oxygen species (ROS) in *SV* . Similarly, histochemical staining using diaminobenzidine (DAB) showed no visible brown spots on either control or salt-treated*SA* leaves (Figure 10A-B). In contrast, *SV* treated with only 50 mM NaCl for 12 days shows a widespread presence of brown spots on the leaf surface (Figure 10C-D).

4 DISCUSSION

In this section, we first discuss the major finding of the up-regulation of PTOX in *SA* and PTOX as a new mechanism to cope up with salt stress, and then we discuss the general physiological role of PTOX.

4.1 Salt stress induced up-regulation of electron flow through the PTOX activity in Spartina

There is a huge difference between *SV* and *SA* regarding their physological response to salt. Here, we found that in *SA,*with time, either a stable or an increase in the K^+/Na^+ was observed (Figure 3E-F). This maintenance or increase in the K^+/Na^+ is a major trait associated with salt tolerance (Shabala & Pottosin, 2014). Na^+tolerance is associated with SOS1 antiporter localized to the root epidermis (Shi et al., 2002). Mostly, halophytes exhibit higher SOS1 abundance (Oh et al., 2009). Therefore, exclusion of Na^+ should also be a mechanism involved in salt-tolerance in *SA.* In addition to this known mechanism of salt tolerance, here our data suggest that under salt, *SA* gained increased salt tolerance through increased electron flow through PTOX.

First, under normal growth conditions, i.e. when there was no salt stress, the NDH-dependent CEF activity was more than two times higher in*SA* than in *SV* under normal condition (Figure 6); however, after NaCl treatment, the NDH activity was enhanced by 2.36 times in*SV* but decreased by about 25% in *SA* , compared to their respective control (Figure 6). After exposure to salt stress, the J-step of OJIP curves was significantly enhanced for *SV* compared to*SA* (data not data). The increased J level is an indicator of a more reduced PQ pool and a more pronounced Q^{-}_{A} (primary electron acceptor of PSII) accumulation under salt stress (Haldimann & Strasser, 1999). This leads to a strong PSII acceptor side limitation and a high PQ pool over-reduction in *SV* compared to *SA* . Furthermore, we found that under salt stress, the level of NPQ was similar between *SA*and *SV,* i.e. the incident light energy was not more dissipated in the form of heat in *SA* , as compared to *SV.* There must be a major source of electron which accept electron in *SA* under salt stress.

Second, experiments using inhibitors suggest that PTOX is a major sink of electrons in *SA* under salt. To test this, we examined the PSII photoinhibition following salt stress in presence of *n* -PG (PTOX inhibitor) or DBMIB (Q_o-binding site of $Cytb_6f$ inhibitor) at atmospheric CO_2(390 µL L^{-1} CO_2) and in presence of 2 or 21% O_2 (Figure 7). Our results revealed that the restriction in electrons flow towards PTOX (*n-PG*) has little effect on the $?_{PSII}$ in *SV* (Figure 7A) but significantly decreased $?_{PSII}$ in *SA* under both normal and even more severely under low O_2 (Figure 7B). This reflects that a proportion of electrons from PSII is sensitive to both to *n* -PG and O_2 (13%, Figure 7B). This provides an evidence that an efficiently operating PTOX in *SA* but not in *SV* under salt stress. In fact, even under non-salt condition, there is a proportion of electron from PSI flow into PTOX driven reactions.

Thirdly, using DBMIB, we observed that in *SA,* as compared to*SV* , under high NaCl treatment, the PSII was less photoinhibited, especially at the presence of 21% O_2 (Fig 7C, D); this is possibly because under severe salt stress, electrons can be used to reduce O_2 in *SA* through PTOX without passing through $Cytb_6f$. Consistent with this possibility, we observed an enhancement in the primary PSII electron transfer rate under salt in the presence of 21% O_2 and saturating CO_2, 2000 μL L^{-1} (Figure 5B). Under 2000 ml·L^{-1} CO_2, the flux through photorespiration minimizes, hence the photorespiration as a major sink for reducing power is minimized. This provides further evidence that PTOX may functions as a major electron sink in *SA* under salt stress. Furthermore, in line with this notation, this enhancement of electron transfer rate was not observed under low O_2(2%) under salt stress (Figure 5A, B). The gene expression and Westernblot analysis (Figure 8) also showed that under salt stress, there were increased amount of PTOX RNA and protein abundance in*SA* , but not in *SV* (Figure 8). Therefore, upon salt stress, the *SA* shows drastically increased electron flow into TPOX. The increase of PTOX levels have also been reported earlier in plants under stress, e.g. exposure of tomato to high light (Shahbazi et al., 2007) or thellungiella to salt stress (Stepien &Johnson, 2009).

4.2 PTOX as a safety valve in *SA* under salt stress to protect photosystems from over-reduction

PTOX is an interfacial membrane protein (Berthold & Stenmark, 2003) attached to the stromal-side of the thylakoid membrane (Lennon et al., 2003). PTOX is involved in the carotenoid biosynthesis (Carol & Kuntz, 2001) and has been implicated in the oxidation of the plastoquinol pool, PQH_2 (Joet et al., 2002). Similar to the increase of PTOX under salt conditions in *SA,* the PTOX levels have been found to increase in plants exposed to abiotic stress such as high temperatures, high light and drought (Quiles, 2006; Diaz et al., 2007; Ibanez et al., 2010), low temperatures and high light (Ivanov et al., 2012), salinity (Stepien & Johnson, 2009) and in alpine plants at low temperature and high UV exposure (Streb et al., 2005; Laureau et al., 2013), implying a generic role of PTOX under stress.

Data from this study provide new evidence for the protective role of PTOX under salt stress. F_o of Chl *a* fluorescence (OJIP) was found to increase in *SV* but was not changed or changed little for *SA* (data not shown). After exposure to salt stress, the J-step of OJIP curves was significantly enhanced for *SV*compared to *SA* (data not shown). The increased J level is an indicator of a more reduced PQ pool and a more pronounced Q^-_A (primary electron acceptor of PSII) accumulation under salt stress (Haldimann & Strasser, 1999). This leads to a strong PSII acceptor side limitation and a high PQ pool over-reduction in *SV* compared to *SA* . In this regard, similar results have been reported by Shahbazi et al. (2007). These authors proved similar effect of high light treatment on the mutant of tomato *ghost* (*gh*) defective in PTOX compared to the control San Marzano (SM) (Shahbazi et al., 2007). The data from this study, together with these earlier studies, suggests that PTOX can oxidize over-reduced PQ pool and hence provides protective roles.

As a reflection of the protective role, *SA* plants grew normally at a moderate salt stress and even survived under NaCl concentrations up to 550 mM NaCl without significant mortality. The Chl content of leaves did not drop significantly, particularly at NaCl concentrations below 250 mM (Figure 4B) and both stomatal conductance (*gs*) and assimilation at atmospheric CO_2 concentrations (A) were maintained (Essemine et al., unpublished data). By comparison, *SV*was unable to survive at NaCl level higher than 100 mM for two weeks; even at NaCl concentrations lower than 100 mM, the Chl content of*SV* dropped drastically by about 42 and 58% after 12 days exposure of *SV* to 50 and 100 mM NaCl, respectively (Figure 4A), concurrent with a dramatic decline in both *gs* and *A*(Essemine et al., unpublished data).

The protective role is clearly shown by changes in the linear electron transfer rates under NaCl treatments. In *SV,* under salt stress, we observed a decrease in linear electron transfer rate (LEF) in*SV,* as shown by the decrease in the g_{ETC}at saturating CO_2, which has a concentration of 2000 μL L^{-1} at either 21% or 2% O_2 levels (Figure 5). Such decrease is common among C_3 species under stress, e.g. drought (Golding & Johnson, 2003), salt (Stepien & Johnson, 2009), and anaerobiosis (Haldimann & Strasser, 1999). In*SA* , in contrast, there was no apparent decrease in LET under salt (Figure 5B); which suggests that the photosystem II in *SA* under stress was well protected. Consistent with these differential capacities to protect photosystem under salt, we observed much higher accumulation of ROS in *SV* compared to *SA* , even though the salt

concentration used to treat *SV* was 50 mM, while that used to treat *SA* was 250 mM (Figure 9, 10). The ROS detected here may include highly reactive singlet oxygen (Kearns, 1971), the superoxide anion radical and hydrogen peroxide (Fridovich, 1997). The severe damage of salt to photosystem in *SV* is also reflected by a swelling in the chloroplast structure for *SV* after exposure to salt (Essemine et al., unpublished data). Altogether, these data suggest that having higher PTOX activity under salt (Figure 8) may contributed to the protection of chloroplast structure and function, as shown by maintenance of the photosynthetic linear electron transfer, chlorophyll a content, and less accumulation of ROS in leaves.

It is worth mentioning here that the protective function of PTOX has been studied earlier through transgenic approaches. However, the data obtained so far from transgenic experiments are still not conclusive. When PTOX from Chlamydomonas reinhardii was transferred into tobacco (Ahmad et al., 2012), it resulted in growth retardation; furthermore, instead of inducing increased resistance to high light, it led to increased vulnerability to high light for tobacco. The ortholog of PTOX in Arabidopsis has also been studied using both mutant and over-expression lines; which however, did not provide evidence for a role of PTOX in the regulation of PQ redox status (Rosso et al., 2006). In tobacco, however, over-expression of PTOX led to increased photoprotection under low light but increased vulnerability under high light, or which the authors suggest that the PTOX can only provide a sufficient photoprotection when the reactive oxygen species generated by PTOX can be effectively detoxified (Heyno et al., 2009). However, the enhanced sensitivity of plant growth to high light was not shown in tobacco over-expressing PTOX from Arabidopsis (Joët et al., 2002). In high mountain species *Ranunculus glacialis* , the rate of the linear electron transfer far exceeds the rate of consumption of electrons for carbon assimilation rate under different temperature and light levels; especially under 21% O_2 and high C_i, suggesting a major role of PTOX in photoprotection (Streb et al., 2005).

4.3 PTOX and NDH-mediated cyclic electron transfer

Under stress conditions, the cyclic electron transfer rate usually increases (Brayton et al., 2006; Shikanai 2007; Takahashi et al., 2013; Strend et al., 2015). In contrast, here we show that in *SA* , which has a great capacity of channeling electrons to PTOX, the rate of cyclic electron transfer rate decreased (Figure 6). This is clearly shown by data from the post-illumination Fo rise (PIR) signal, which was used here to assay NDH (Burrows et al., 1998). Using this method, we found a stark contrast in the responses of NDH-dependent CEF and PTOX to salt stress between species (*SA* and *SV*). In *SV* , the strong stimulation of NDH-dependent CEF following salt stress (236%) was concurrent to a nearly stable PTOX level (Figs. 6 and 8). However, in *SA* , we observed a decline in the NDH-dependent CEF (Figure 6) together with an increase of PTOX expression levels, which was up-regulated by up to 4 times compared to the control as assessed by both RNA-expression abundance and protein abundance (Figure 8).

Our finding of this negative relationship between PTOX and NDH-CEF is in line with a number of earlier reports, e.g. Ahmad et al. (2012) demonstrated a dramatic decline in NDH activity in tobacco expressing PTOX from green algae (Cr-PTOX1); PTOX competes efficiently with CEF for plastoquinol (PQH2) in the CRTI-expressing (carotene desaturase) lines (Galzerano et al., 2014); Joët et al. (2002) also showed a decrease in NDH-dependent CEF in tobacco transgenic lines expressing PTOX from Arabidopsis. The activity of cyclic electron transfer is regulated by an array of mechanisms, including redox status (Lasano et al., 2001; Breyton et al., 2006; Takahashi et al., 2013), H_2O_2 (Strand et al., 2015), metabolite levels (Livingston et al., 2010), Ca signaling (Terashima et al., 2012; Lascano et al., 2003), and even phosphorylation of NDH components (Lascano et al., 2003). It is likely that NaCl induced differential changes in the NDH and PTOX, though mechanism is complexly unknown. It is possible that some internal signals from chloroplast, such as redox status of chloroplast electron transfer chain, or particular compound in the photosynthetic carbon metabolism, or even H_2O_2, might differentially regulate PTOX and NDH-CET. Mechanisms how PTOX and NDH-CET were differentially regulated under NaCl needs further elucidation. It is worth mentioning here that *SA* has been used as a model halophyte grass species to study adaptation to plants to salt stress and to mine salt stress-responsive genes (Subudhi & Baisakh, 2011). Several ealier studies have demonstrated the utility of genes from this halophyte to improve crop salt tolerance (Baisakh et al. 2012; Karan & Subudhi,

2012a, b). Therefore, elucidation of how PTOX and NDH-CET respond under NaCl to protect photosystem and leaf functioning can help develop new strategy to protect photosystems under salt stress.

Abbreviations : A , photosynthetic rate; CAT, catalase; CEF, cyclic electron flow; Chl, chlorophyll; DAB, 3, 3' diaminobenzidine; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-p- benzoquinone; ETC, electron transport chain; FI, fluorescence induction;gs , stomatal conductance; F_o, minimum fluorescence (PSII RCs open); F_m, maximum fluorescence (PSII RCs close); g_{ETC}, conductance of the electron transfer chain; n -PG, n -propyl gallate (n -PG, 3,4,5-trihydroxy-benzoic acid-n-propyl ester); NBT, nitroblue tetrazolium; NDH, NAD(P)H dehydrogenase; NPQ, non-photochemical quenching; PQ, plastoquinone; PQH_2, plastoquinol; PTOX, plastid terminal oxidase; P_{700}, photosystem I reaction center; ROS, reactive oxygen species; SP, saturating pulse; SOD, superoxide dismutase; SV , *Setaria viridis* ; SA ,*Spartina alterniflora* .

SUPPORTING INFORMATION

This document includes two Tables, one video and some other genes sequences and primers.

Disclosures

The authors have no conflicts of interest to declare.

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Figures legends

FIGURE 1 Schematic representation showing the experimental setting of Li-cor 6400 together with Dual-PAM-100, through a chamber holding on the emitter-detector system of the PAM, for controlling CO_2 level (390 or 2000 µL L^{-1}) and using an oxygen source equipped with an oxymeter to adjust oxygen flux from the appropriate O_2 source (2 or 21%) to the chamber and accordingly be able to monitor the CO_2 and O_2 concentrations in the chamber during measurements. See also video for setting. IRGA and PAM mean infrared gas analyzer and pulse amplitude modulation, respectively.

FIGURE 2 A 'spider plot' of selected parameters derived from the chlorophyll fluorescence OJIP curves for Setaria (left column, A-C) and Spartina (right column, D-F) treated with 0, 50 and 100 mM (A-C) and 0, 100, 250, 400 and 550 mM (D-F) NaCl for 12 days. All data of JIP test parameters were normalized to the reference 0 mM NaCl and each variable at the reference was standardized by giving a numerical value of the unit (1).

FIGURE 3 Changes in leaf Na^+ (A-B), K^+ (C-D) contents and K^+/Na^+ ratio (E-F) over time in Setaria (A, C and E) and Spartina (B, D and F). One-month old Setaria and 2 month-old Spartina were exposed to salt for up to 2 weeks. Plants were subjected to: 0, 50, and 100 mM NaCl for Setaria and 0, 100, 250, 400, and 550 mM NaCl for Spartina. Data represent the means of 4 to 5 replicates ±SE.

FIGURE 4 The effect of salt on the leaf total chlorophyll content in Setaria (A) and Spartina (B). One month-old Setaria and two month-old Spartina were exposed to different NaCl levels as described in Fig. 2. Leaves were collected 12 days after initiating salt treatment to determine chlorophyll concentration. For chlorophyll each data bar represents the mean of at least 10 replicates ± SE. Fast- and slow-relaxing components of NPQ (NPQ_f and NPQs) in leaves of Setaria (C) and Spartina (D) exposed to to 0 and 100 (C) or 0 and 400 mM NaCl (D). Measurements were carried out 16 days after initiating salt treatment at 25°C in the presence of 390 µL L^{-1} CO_2. Leaves were illuminated with 800 µmol m^{-2} s^{-1} actinic light. Each data bar represents the means of at least 6 replicates ±SE.

FIGURE 5 Oxygen dependence of electron transport: ETR_{II} (A-B), P_{700} oxidation ratio (C-D),g_{ETC} (E-F), and ETR_I (G-H), measured in leaves of Setaria (left column, A, C, E and G) and Spartina (right column, B, D, F and H) endured NaCl (triangles): 100 mM for Setaria and 250 mM for Spartina. Control plants (circles) were maintained in a NaCl-free medium. The measurements were performed, 12 days after initiating salt treatment, under saturating CO_2 (2000 µL L^{-1}), at 25°C and in the presence of 21% (open symbols) or 2% (closed symbols) oxygen. Each data point represents the means of at least 6 replicates ±SE.

FIGURE 6 NDH-dependent CEF pathway assessed as the post-illumination F_o rise in plants grown on either salt-free medium (ctrl) or subjected to 50 or 250 mM NaCl for Setaria and Spartina, respectively, during 12 days. The post-illumination F_o rsie was recorded in the dark after switching off 5 min illumination with actinic light (325 µmol $m^{-2}s^{-1}$). Each data bar represents the means of at least 10 replicates taken on different leaves ±SE.

FIGURE 7 Effects of *n* -PG and DBMIB on PSII photochemical efficiency Φ_{PSII} measured in leaves of either Setaria (A and C) or Spartina (B and D) detached from plants subjected to: 0 and 50 (for *SV*) ; or 0 and 250 mM NaCl (for *SA*). Measurements were carried out 12 days after initiating salt treatment at 25°C in the presence of 390 µL L^{-1} CO_2. Leaves were illuminated with 800 µmol $m^{-2}s^{-1}$ red actinic light. Leaves were vacuum infiltrated with water (white bars) or with 5 mM *n* -PG (A-B) or 50 µM DBMIB (C-D) in the presence of 21% (gray bars) and 2% (black bars) oxygen. Each data point exhibits the means of at least 6 replicates ±SE.

FIGURE 8 Effect of salt treatment on PTOX protein expression (A) and the PTOX gene expression level assessed by q-PCR analysis (B) in leaves of Setaria and Spartina subjected to 0 and 50 (for *SV*) or to 0 and 250 mM NaCl (for *SA*). For protein expression (A), leaves from control and salt-treated plants were collected 12 days after initiating salt treatment for immune-detection after SDS-PAGE, separation of 29 µg protein from the thylakoid membrane samples, and electrophoretic transfer to nitrocellulose membrane. Western-blot band size was quantified by TanonImage technology software. For gene expression level (B), leaves from control and salt-treated plants for 12 days were collected and immediately stored in liquid nitrogen for RNA isolation and purification using PureLink RNA Mini Kit Invitrogen (see materials and methods). The synthesized cDNA was used for the q-PCR analysis of PTOX. Data points represent the mean of around five replicates for western SDS-PAGE ±SE and 6 replicates for qRT-PCR. Insert of panel A shows typical bands from an original blot, loaded on an equal protein basis.

FIGURE 9 Histochemical staining of Setaria (C-D) and Spartina (A-B) leaves obtained from control untreated (A and C) or salt treated (B and D) for Setaria at 50 mM and Spartina at 250 mM during 12 days with 6 mM NBT (nitroblue tetrazolium). Dark-blue staining reveals the interaction of superoxide radical ($\cdot O^-_2$) with NBT (500 µmol m^{-2} s^{-1}) in leaves following salt stress treatment.

FIGURE 10 Histochemical staining of Setaria (C-D) and Spartina (A-B) leaves obtained from control untreated (A and C) or salt treated (B and D) for Setaria at 50 mM and Spartina at 250 mM during 12 days with 5 mM DAB (diaminobenzidine). Brown spots reflect the interaction of hydrogen peroxide (H_2O_2) with DAB under light (500 µmol m^{-2} s^{-1}) in leaves following salt stress treatment.