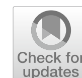
Planta (2020) 251:34
https://doi.org/10.1007/s00425-019-03325-w

ORIGINAL ARTICLE



Transcriptional, metabolic and DNA methylation changes underpinning the response of *Arundo donax* ecotypes to NaCl excess

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Received: 27 February 2019 / Accepted: 6 December 2019 / Published online: 17 December 2019
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Abstract

Main conclusion ***Arundo donax*** **ecotypes react differently to salinity, partly due to differences in constitutive defences and methylome plasticity.**

Abstract *Arundo donax* L. is a C3 fast-growing grass that yields high biomass under stress. To elucidate its ability to produce biomass under high salinity, we investigated short/long-term NaCl responses of three ecotypes through transcriptional, metabolic and DNA methylation profiling of leaves and roots. Prolonged salt treatment discriminated the sensitive ecotype 'Cercola' from the tolerant 'Domitiana' and 'Canneto' in terms of biomass. Transcriptional and metabolic responses to NaCl differed between the ecotypes. In roots, constitutive expression of ion transporter and stress-related transcription factors' genes was higher in 'Canneto' and 'Domitiana' than 'Cercola' and 21-day NaCl drove strong up-regulation in all ecotypes. In leaves, unstressed 'Domitiana' confirmed higher expression of the above genes, whose transcription was repressed in 'Domitiana' but induced in 'Cercola' following NaCl treatment. In all ecotypes, salinity increased proline, ABA and leaf antioxidants, paralleled by up-regulation of antioxidant genes in 'Canneto' and 'Cercola' but not in 'Domitiana', which tolerated a higher level of oxidative damage. Changes in DNA methylation patterns highlighted a marked capacity of the tolerant 'Domitiana' ecotype to adjust DNA methylation to salt stress. The reduced salt sensitivity of 'Domitiana' and, to a lesser extent, 'Canneto' appears to rely on a complex set of constitutively activated defences, possibly due to the environmental conditions of the site of origin, and on higher plasticity of the methylome. Our findings provide insights into the mechanisms of adaptability of *A. donax* ecotypes to salinity, offering new perspectives for the improvement of this species for cultivation in limiting environments.

Keywords Abscisic acid · DNA methylation · Giant reed · Proline · ROS · Salinity

Abbreviations

MDA Malondialdehyde
MSAP Methylation-sensitive amplification polymorphism
TF Transcription factor

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00425-019-03325-w) contains supplementary material, which is available to authorized users.

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Introduction

Global warming is strongly exacerbating salinity of arable lands, since water evaporation causes over accumulation of sodium (Na^+) and chloride (Cl^-) in the soil (Ismail et al. 2014). Giant reed (*Arundo donax* L.), a perennial weed that is increasing cultivated for ligno-cellulosic biomass production in temperate climates, possesses large environmental plasticity, being able to survive in suboptimal conditions and to give high yields even in harsh environments. The ability of *A. donax* to tolerate diverse abiotic stresses, and mainly salt stress, has been reported in several studies (Lewandowski et al. 2003; Papazoglou 2007; Nackley and Kim 2015; Sánchez et al. 2015; De Stefano et al. 2017), but the investigation at the genomic and metabolic level are just at the beginning (Malone et al. 2017). Since genetic

diversity in this species is reportedly scarce (Ahmad et al. 2008; Mariani et al. 2010; Saltonstall et al. 2010; Pilu et al. 2014; Guarino et al. 2019), also because of its obligatory asexual mode of reproduction, it is still matter of debate whether specimens of *A. donax* collected in different environments, defined as ecotypes (Haworth et al. 2017c), may have different physiological adaptation to stress in function of genetic differences or of the original living conditions.

Excess salinity reduces soil water potential, thus impacting on plant water uptake and resulting in water deficiency and osmotic stress. Moreover, Na^+ and, to a lesser extent, Cl^- are toxic to plant cells, causing reduced photosynthesis, oxidative damage, nutritional imbalance, and metabolic changes (Hasegawa et al. 2000; Negrao et al. 2017). Perception of high ionic concentrations activates stress pathways that overhang the normal developmental process and may impair plant growth and productivity or even lead to plant death. Salinity tolerance is a complex trait, triggered by several genes involved in osmoregulation, exclusion of toxic ions and tissue tolerance and controlled by different genetic pathways (DeRose-Wilson and Gaut 2011). A key component of osmotic regulation is the SOS (Salt Overly Sensitive) pathway (Qiu et al. 2002; Yang et al. 2009). Under salt stress, the protein kinases SOS2 and SOS3 activate a Ca^{2+}-dependent signalling cascade, which promotes Na^+ efflux from the cells by SOS1 (Na^+/H^+ antiporter) as well as abscisic acid (ABA) signalling involved in root to shoot communication of salinity stress (Munns and Tester 2008; Zhu 2016). Another crucial salt tolerance mechanism is mediated by the high-affinity plasma membrane K^+ channel (HKT), which alleviates Na^+ toxicity by promoting Na^+ efflux and K^+ uptake in the cytoplasm (Davenport et al. 2007; Kobayashi et al. 2017). To avoid negative effects on cell metabolism, excess sodium in the cytoplasm is also directed to the vacuole by the tonoplast Na^+/H^+ antiporter (NHX), whose activity is positively regulated by SOS2. Lately, several members of the NHX family emerged to have an equally important role as H^+/K^+ exchanger, thus contributing to maintain higher level of cytosolic K and to preserve K-dependent metabolic processes, such as protein synthesis (Moller et al. 2009).

Besides maintenance of ion homeostasis, stress signals activate ABA-dependent and ABA-independent transcription factors (TFs), mainly of the dehydration-responsive element binding (DREB), NAC, WRKY and MYB families (Golldack et al. 2014), that assist the above-reported pathways but also trigger and orchestrate transduction pathways tightly linked to cell metabolism and adaptive mechanisms under high salinity, including accumulation of antioxidant molecules (Sairam and Tyagi 2004; Gill and Tuteja 2010). Salt stress induces reactive oxygen species (ROS), such as O_2^-, $^{\bullet}OH$, $^{\bullet}O_2^-$ and H_2O_2 (Price et al. 1989; Moran et al. 1994; Mittler 2002), which cause oxidative damage of membranes and macromolecules. To scavenge these toxic compounds, plants have evolved robust antioxidant defences through enzymatic mechanisms, including superoxide dismutase (SOD), whose H_2O_2 production is further scavenged by catalase (CAT) activity, and non-enzymatic mechanisms, e.g. accumulation of ascorbic acid, glutathione, which is regenerated by glutathione reductase (GR), and polyphenolic compounds.

Recently, epigenetic mechanisms such as histone modifications, production of small non-coding RNAs, and DNA methylation have been reported to be central in adaptation processes when plants are challenged by stressful environmental cues (Deinlein et al. 2014; Ferreira et al. 2015) and were proposed to underlie the remarkable phenotypic plasticity of *A. donax* (Guarino et al. 2019). Growing evidences indicate DNA cytosine methylation as a crucial mechanism helping individuals to cope with abiotic stress, including salinity, through regulation of gene expression. For example, mangrove plants growing in contrasting natural habitats, such as periodic drought and hyper-saline soils, differed with respect to cytosine methylation despite little genetic differentiation (Lira-Medeiros et al. 2010). However, the involvement of methylation/de-methylation processes in salinity stress is still poorly understood.

Here, we aimed to analyse the response under short- and long-term salt treatment of three *A. donax* ecotypes displaying different sensitivity to NaCl (De Stefano et al. 2017). We characterised local (root) and distal (leaf) morpho-physiological parameters and gene expression of salt stress-responsive key genes and the gain/loss of metabolites notoriously involved in salt tolerance, such as proline, ABA, malondialdehyde (MDA) and antioxidants. Moreover, to understand whether epigenomic changes may have contributed to the observed variability in the NaCl response of these *A. donax* ecotypes, we characterised the root and leaf changes in total DNA methylation during short and long exposure to NaCl stress. Our results highlighted different morpho-physiological, transcriptional and metabolic responses to NaCl of the three ecotypes, which may account for their different levels of NaCl tolerance. We found that giant reed ecotypes were able to rapidly change DNA methylation (either reducing or increasing) upon stress imposition, displaying differential methylome flexibility.

Materials and methods

Plant growth and experimental design

The plant genetic material screened in this paper was collected in different hydrogeological basins in the Italian Campania region (Supplementary Table S1). The sampled stand of reeds from each collection site, namely 'Canneto',

'Cercola' and 'Domitiana', was considered to be one ecotype. For each experiment, plant material was re-collected in each original site. The data presented correspond to one representative experiment, which was repeated three times with similar results.

Portions of reeds of about 30 cm were put in large plastic trays filled with weekly refreshed tap water in a temperature-controlled greenhouse of the CNR-IBBR Portici (27 °C day/24 °C night). After about 40 days, rooted sprouts produced from the nodes were collected and transferred in vermiculite-filled black-seed trays (4 × 6 cm pots) in hydroponic float system, blowing air with aquarium aerators. Plants were kept in half-strength modified Hoagland nutrient solution (1.23 mS cm^{-1} electrical conductivity, EC) for about 30 days, followed by four additional days in full-strength-modified nutrient solution (electric conductivity of 1.92 mS cm^{-1}) (Hoagland and Arnon 1950) as described by De Stefano et al. (2017). The obtained plants were then randomly divided between control and NaCl treatments. For the latter, NaCl was added stepwise in 3 consecutive days up to 150 mM (electric conductivity of 18.8 mS cm^{-1}) and maintained for 21 days. A completely randomized block design, with 12-replica plants per thesis (ecotype × treatment) was adopted.

For RNA extraction and biochemical analyses, *A. donax* leaves and roots were collected after 2 and 21 days of exposure to 0 or 150 mM NaCl.

Evaluation of salt tolerance

Arundo donax response to NaCl stress was evaluated by checking six biometric parameters after the 21-day NaCl treatment, i.e. stem height, leaf number, fourth leaf width, main root length, shoot and root fresh weight (FW). All values are presented as mean ± SE. For mean comparison and statistical significance, pairwise *t* test was performed between stressed and control plants within each ecotype.

Leaf greenness was measured using a portable chlorophyll meter (SPAD 502 Plus Meter, Konica Minolta, Tokyo, Japan) in exposed to 2 and 21 days of 0 mM (control) or 150 mM NaCl (stress treatment) treatment. SPAD readings were the average of five measurements from the middle of the second or third last fully collared leaf for each plant. For each ecotype/treatment, SPAD values are expressed as mean ± SD of four plants.

A stress susceptibility index (SSI) was calculated according to the following relationship $[(1-(Ys/Yp))]/[(1-(Xs/Xp))]$ (Sánchez et al. 2015), where Ys, Yp, represent parameter under stress, parameter under non-stress conditions for each ecotype, and Xs and Xp parameter mean under stress and parameter mean under non-stress conditions for all ecotypes. The results for stem height, leaf number, fourth leaf width, and shoot FW, were averaged and used to classify the ecotypes in terms of tolerance/sensitivity to NaCl stress.

RNA extraction and qRT-PCR

Samples were ground in liquid nitrogen and total RNA was extracted from 100 mg of tissue using the RNeasy kit (Qiagen). After visual check of RNA integrity on agarose gels and quantification using a NanoDrop ND-8000 spectrophotometer (Fisher Scientific, Waltham, MA, USA), 1 µg of total RNA was reversed transcribed using the QuantiTec Reverse Transcription Kit (Qiagen), according to the manufacturer's instructions. Real-time qRT-PCR was performed with Platinum® SYBR® Green qPCR Super Mix (Thermo Fisher Scientific) in a ABI7900 HT (Thermo Fisher Scientific). Each PCR reaction (20 µL) contained 10 µL real-time qRT-PCR Mix, 4 µL of a 1:25 dilution of cDNA and 0.25 µM of each specific primer. The thermal cycling conditions were 50 °C for 2 min, 95 °C for 2 min, followed by 40 cycles of 15 s at 95 °C and 30 s at 60 °C. PCR product melting curves were analysed for the presence of a single peak. All reactions were performed on biological triplicates and technical duplicates and fold change measurements calculated with the $2^{-\Delta\Delta CT}$ method (Pfaffl 2001, 2004). The 'Cercola' ecotype in control condition was used as internal calibrator. Gene expression was normalized on the stably expressed *A. donax* elongation factor gene (Poli et al. 2017). For primer design, identification of putative *A. donax* stress-related genes was based on homology searches. Well-known *Arabidopsis thaliana* stress-responsive genes from the Arabidopsis Stress Responsive Gene Database available at http://srgdb.bicpu.edu.in/ (ASRGDB; Borkotoky et al. 2013) were used to identify the putative homologs and to assess transcript homolog coverage and sequence conservation across species phylogenetically related to *A. donax* by searching the Phytozome database (Goodstein et al. 2012) with BLASTx with an *E* value cut-off of 1×10^{3} and sequence coverage greater than 50% of the subject length. The retrieved sequences were then used to run TBLASTN homology searches against the *A. donax* transcriptome available in NCBI at https://www.ncbi.nlm.nih.gov/nuccore/?term=Arundo+donax with an *E* value cut-off of 1×10^{3} and sequence identity greater than 50%. Whenever possible, the giant reed sequences were also verified in other transcriptomic databases (Sablok et al. 2014; Barrero et al. 2015; Fu et al. 2016; Evangelistella et al. 2017). The primers designed on the retrieved *A. donax* sequences and used for real-time qRT-PCR are provided in Supplementary Table S2, together with the relative homologs of *A. thaliana* and monocot species.

Proline and free ABA determination

Leaf samples (250 mg) were homogenized in liquid nitrogen and extracted with acidic ninhydrin reagent following a procedure previously described by Claussen (2005). Proline concentration was determined from a standard curve and calculated on a fresh weight basis (µmol proline g^{-1} FW) using three biological and three technical replicates.

For ABA extraction, freeze-dried samples (150 mg) were homogenized in liquid nitrogen and incubated in 2 mL of water overnight at 4 °C. After sample centrifugation at 9279*g* for 10 min, quantitative free ABA determination was performed on three biological triplicates and technical duplicates by the competitive ELISA Phytodetek ABA test kit (Agdia-Biofords, Evry, France) following the provider's protocol. Colour absorbance was read at 405 nm using a plate auto-reader (1420 Multilabel Counter Victor3TM, PerkinElmer) and free ABA content was expressed in nmol g^{-1} FW.

Determination of total antioxidant activity (TEAC) and lipid peroxidation

Leaf samples were homogenized (250 mg) and extracted twice with water (hydrophilic antioxidants). The residue was re-extracted twice in acetone (lipophilic antioxidants). The TEAC assay was performed as previously described by Butelli et al. (2008). Results were expressed as mmol Trolox kg^{-1} FW. TEAC was measured on three biological replicates and three technical replicates.

Malondialdehyde content was quantified by the thiobarbituric acid-reactive substance assay (Heath and Packer 1968). Briefly, 100 mg of freeze-dried leaf tissue was homogenized in liquid nitrogen from control and salt-treated plants, and vortexed, for few seconds, in 5 mL of 5% (w/v) trichloroacetic acid. The homogenate was centrifuged at 12,000*g* for 10 min at room temperature. Supernatant was mixed with an equal volume of thiobarbituric acid [0.5% in 20% (w/v) trichloroacetic acid] and the mixture was then boiled for 25 min at 100 °C. Then, centrifugation for 5 min at 7500*g* was performed to clarify the solution. Absorbance of the supernatant was measured at 532 nm. MDA equivalents were calculated by the extinction coefficient of 155 mM^{-1} cm and expressed as nmol MDA g^{-1} FW.

Methylation-sensitive amplified polymorphism (MSAP) profiling and data analysis

Total DNA was extracted from 50 mg of leaves using the Qiagen Plant DNeasy Kit according to the manufacturer's instructions (Qiagen). DNA quality and integrity were checked by the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific) and fluorimeter (Qubit 2.0, Thermo Fisher Scientific). The methylation pattern at the 5′-CCGG sites was analysed using the methylation-sensitive amplification polymorphism (MSAP) technique, which employs the methylation-sensitive isoschizomers, HpaII and MspI. The MSAP protocol developed by Albertini and Marconi 2014 was followed. For selective amplifications, one FAM-labelled EcoRI primer (EcoRI-CCA) and one HEX-labelled EcoRI primer (EcoRI-CAA) were combined with four HpaII/MspI primers for a total of four primer combinations (EcoRI-CCA/HpaII-AAC; EcoRI-CCA/HpaII-ACT; EcoRI-CAA/HpaII-ACC; EcoRI-CCA/HpaII-AGG). The obtained profiles were visualized as band patterns on 2% agarose gel and as electropherograms with the ABI PRISM® 3130 DNA Analyzer system (Thermo Fisher Scientific). Electropherograms' size calibration was performed with the molecular weight ladder GenScan® 500 ROX™ Size Standard (Thermo Fisher Scientific) and the MSAP peaks were detected using the Peak Scanner® software (Thermo Fisher Scientific). We scored markers as present when peak intensity exceeded 30 and discarded all loci showing a signal in a negative control. To have reproducible and clear banding patterns, each amplification was repeated at least three times, and only peaks showing consistent amplification were considered.

Methylation-sensitive amplification polymorphism profiles were recorded as 1/0 binary matrices, where 1 indicates the presence and 0 the absence of a given fragment. The resultant code describes the presence/absence of each fragment (represented by an amplicon) in the EcoRI/HpaII and EcoRI/MspI digests of a single sample. Four permutations (hereafter called pattern types) are expected, depending on the ability of either enzyme to cut at their recognition sequence. In particular, pattern type I (HpaII = 0/MspI = 0) could be caused either by restriction target absence due to a mutated site when genetically distinct samples are compared, or inhibition of digestion with both enzymes at a fully (both strands) methylated CCGG site when another sample shows the presence of a fragment at that position (Schulz et al. 2013; Fulneček and Kovařík 2014). Therefore, the 0–0 profile is not informative and was excluded from the analysis to avoid the noise produced by confounding the effects of mutation and methylation. Pattern type II (HpaII = 0/MspI = 1) reflects hemi- (only one strand) or full methylation of the internal cytosine, where the band is present in the MspI, but absent in HpaII sample; pattern type III (HpaII = 1/MspI = 0) corresponds to hemi-methylation of the external cytosine sites, in which the amplicon is present only in the HpaII, but not in the MspI sample; pattern type IV (HpaII = 1/MspI = 1) reflects un-methylated sites in which a fragment is detected in both the HpaII- and the MspI-digested samples (Supplementary Fig. S1). In agreement with Karan et al. (2012), 16 banding patterns between control and salinity stress in leaf and root of the three ecotypes were observed to find out the changes in cytosine

methylation patterns under salinity stress. In particular, the patterns A–D represented monomorphic class with no methylation changes, the patterns E–J indicated hypomethylation events, while possible hypermethylation induced by salt stress was represented by the patterns K–P. The methylation ratio (MR) parameter was measured to better estimate the predominant event between methylation and de-methylation and calculated as the ratio between the number of fragments showing hyper- and hypo-methylation events according to the following formula: MR = (patterns K–P)/(patterns E–J) (Aversano et al. 2013).

Statistical analysis

The Student's *t* test ($P \leq 0.05$) was used to compare values of the biometric variables of treated versus control samples within each ecotype. qRT-PCR, biochemical and SPAD data were processed by univariate two-way analysis of variance (ANOVA) (treatment × genotype) and mean separation was performed through the Duncan multiple range test, with critical *P* value set at 0.05. All analyses were performed using Sigma Plot Software (Systat Software Inc.).

Results

Growth response of *A. donax* ecotypes under NaCl excess

To understand whether differential response of *A. donax* ecotypes to saline stress could be associated to distinct morpho-physiological, transcriptional, metabolic and DNA methylation level cues, the three ecotypes 'Canneto', 'Cercola' and 'Domitiana' were selected from a larger collection in reason of their different response to salt stress (De Stefano et al. 2017) and treated for 2 and 21 days with 150 mM NaCl. The 21-day prolonged NaCl treatment did not result in plant mortality or severe stress symptoms, since no leaf senescence was detected by visual observation (Supplementary Fig. S2) or decrease in chlorophyll content, expressed as SPAD value (Supplementary Fig. S3). However, the three ecotypes were separated in terms of shoot and root growth. 'Canneto' and 'Domitiana' aboveground growth was not affected by the 21-day NaCl treatment, while 'Cercola' showed a significant decrease in leaf number, stem height and shoot fresh weight (Table 1). Root biomass under salt stress in 'Canneto' and 'Cercola' did not differ from the respective control plants, whereas 'Domitiana' showed stimulated growth, with significantly higher root FW and length than unstressed plants (Table 1).

Based on the ability to maintain plant growth and aerial biomass production under salinity stress, we calculated the stress susceptibility index (SSI) to discriminate the *A. donax* ecotypes, with higher SSI indicating lower tolerance to salt stress. Results classified 'Cercola' as the less tolerant ecotype (SSI = 1.66), 'Canneto' as intermediate (SSI = 1.15) and 'Domitiana' as the most tolerant, with the lowest SSI (SSI = 0.48).

Transcriptional response to NaCl in *A. donax* leaves and roots

Expression profiling of key genes, known to be crucial for maintenance of Na^+/H^+ homeostasis and regulation of transcription under salinity, indicated that the osmotic stress signal is perceived by leaves already after 2 days of NaCl treatment, before Na^+ and Cl^- could have accumulated to toxic levels, and highlighted transcriptional differences between the three *A. donax* ecotypes, indicative of different responses to salt (Fig. 1). However, the largest and most striking transcriptional differences between the ecotypes were observed in control conditions, where intrinsically higher expression levels of most of the analysed genes were detected in 'Domitiana' and, to a lower extent, 'Canneto'. After 21 days of

Table 1 Effects of long-term (21 days) 150 mM NaCl treatment on growth of three *A. donax* ecotypes

Ecotypes	Growth parameters					
	Fourth leaf width (cm)	Leaves (n)	Shoot height (cm)	Shoot FW (g)	Root length (cm)	Root FW (g)
Control						
Canneto	11.67 ± 1.41	7.50 ± 1.05	24.08 ± 5.59	5.40 ± 1.57	24.17 ± 12.92	4.05 ± 1.09
Cercola	13.28 ± 1.61	8.60 ± 1.34	23.00 ± 2.55	7.41 ± 2.16	27.30 ± 10.03	4.50 ± 0.69
Domitiana	12.64 ± 1.25	8.13 ± 1.81	24.56 ± 4.61	5.83 ± 2.26	24.50 ± 6.07	3.02 ± 1.19
150 mM NaCl						
Canneto	11.29 ± 1.64	6.57 ± 1.27	18.31 ± 2.31	4.05 ± 1.10	30.13 ± 9.46	3.67 ± 1.77
Cercola	12.87 ± 2.59	5.43 ± 0.53**	16.21 ± 3.49**	4.51 ± 0.65*	37.86 ± 14.87	4.10 ± 1.78
Domitiana	12.38 ± 1.72	7.42 ± 1.16	23.94 ± 3.45	3.03 ± 1.19	36.88 ± 10.47*	4.5 ± 1.78*

Values are expressed as mean of biological replicates ($n = 12$) ± SE. Within each ecotype, significant differences between control and NaCl-stressed plants by the Student's *t* test are indicated by * ($P \leq 0.05$) or ** ($P \leq 0.001$)

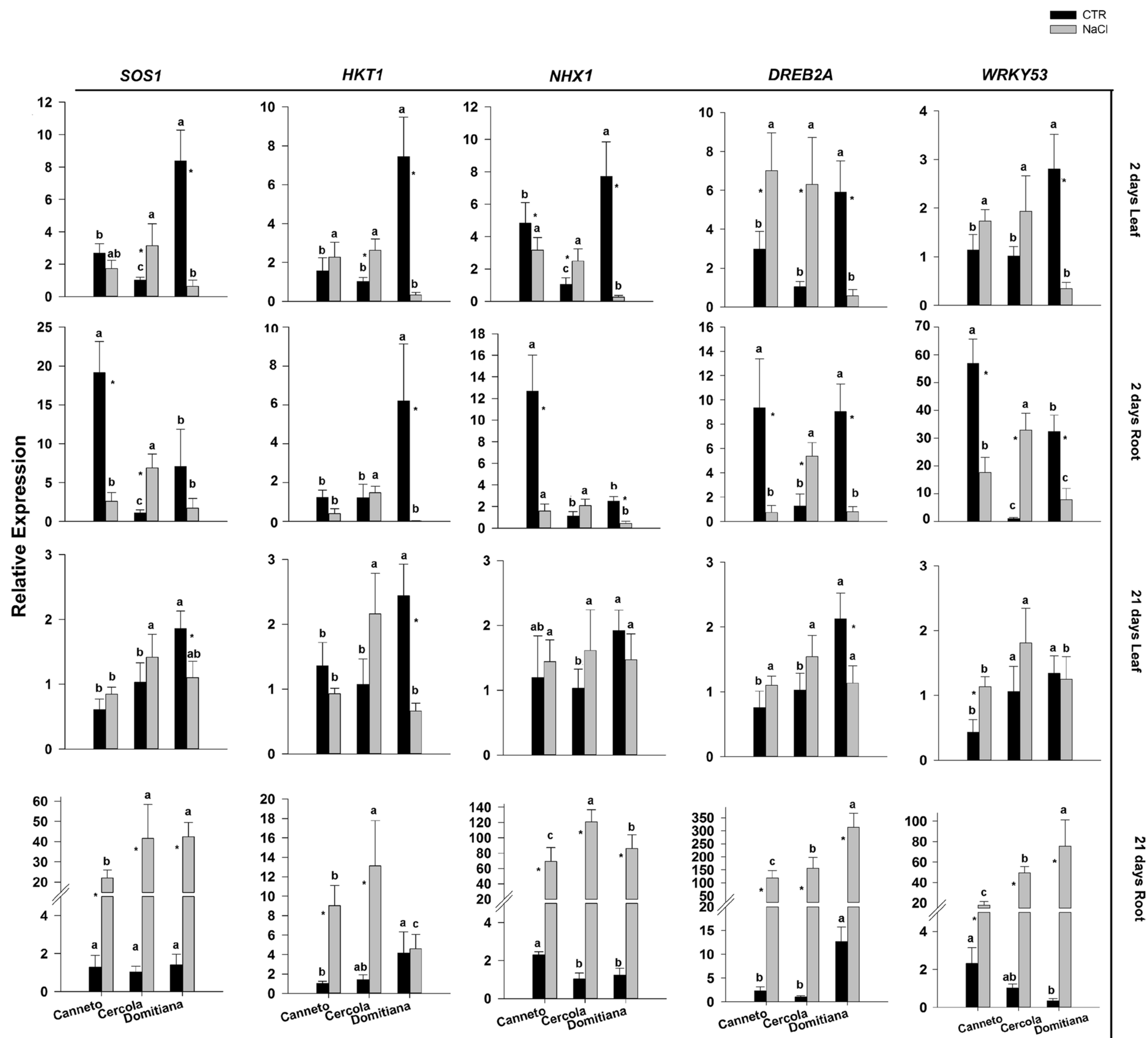

Fig. 1 Transcriptional profiling of key genes related to Na^+/H^+ homeostasis and regulation of gene expression in leaves and roots of three *A. donax* ecotypes after 2 and 21 days of 150 mM NaCl treatment. qRT-PCR results are expressed as fold changes relative to the relevant unstressed control of the ecotype 'Cercola'. Values are the mean ± SD of three technical replicates from three biological samples. Within each treatment, means followed by the same letter are not statistically different (Duncan's test; $P \leq 0.05$). Within each ecotype, differences between NaCl-treated and control plants indicated by * are significant (Duncan's test; $P \leq 0.05$)

NaCl treatment, in leaves, significant differences between ecotypes were detected almost exclusively in 'Cercola', and in roots, the three ecotypes showed strong transcriptional activation of nearly all tested genes (Fig. 1).

Adjustment to ionic and osmotic stress

After 2 days, the Na^+ transporters *SOS1*, *HKT1* and *NHX1* showed almost unchanged or diminished transcriptional response to NaCl treatment in leaves of 'Canneto', whereas in 'Cercola', significant increased expression levels were measured as compared to unstressed leaves (Fig. 1). 'Domitiana', had a remarkably, almost eight times, higher transcription of these genes in control leaves respect to the reference (unstressed 'Cercola' leaves) and a significant decrease following salt stress (Fig. 1). In roots, 'Domitiana', as well as 'Canneto', showed higher expression of the three transporter genes under control conditions and down-regulation following salt stress (Fig. 1). Similar to what detected in leaves, 'Cercola' unstressed roots had lower expression levels of the

three genes, which significantly increased upon salt treatment only for *SOS1*.

Following prolonged NaCl treatment (21 days), the expression levels of ion transporters showed no significant changes in leaves of ‘Canneto’ and ‘Cercola’ compared to the controls, though in the latter ecotype, they showed a trend towards over-expression (Fig. 1). In unstressed leaves of ‘Domitiana’, expression of the three genes was higher than the other two ecotypes, decreasing under NaCl treatment for *SOS1* and *HKT1*. In roots, a strong induction of these genes (20–40 times increase for *SOS1* and 8–10 times increase for *HKT1*) was detected after 21 days of salt stress in the three ecotypes, except for *HKT1* in ‘Domitiana’, which was not significantly different from the control (Fig. 1).

Transcriptional reprogramming

Transcriptional profiling of *A. donax* at 2 and 21 days showed lower expression levels of DREB2A and WRKY53 TF genes in ‘Canneto’ and ‘Cercola’ than in ‘Domitiana’ unstressed leaves (Fig. 1). In response to the 2-day NaCl treatment, leaf expression of DREB2A increased significantly of almost 3–6 times in ‘Canneto’ and ‘Cercola’, respectively, whereas WRKY53 was not significantly different from the controls. In ‘Domitiana’ leaves, both genes were down-regulated by salt treatment. At 2 days, unstressed ‘Cercola’ roots showed lower expression of both TFs than ‘Canneto’ and ‘Domitiana’, but a significant up-regulation following salt treatment. On the contrary, DREB2A and WRKY53 expression was strongly down-regulated in ‘Canneto’ and ‘Domitiana’ roots at this time point. At 21 days, DREB2A and WRKY53 expression increased in ‘Canneto’ and ‘Cercola’ leaves, though significantly only for ‘Canneto’ WRKY53 (Fig. 1). In ‘Domitiana’, TF genes were down-regulated or did not change significantly. After prolonged NaCl treatment, a strong induction of DREB2A and WRKY53 (almost 20–30 times and 10–80 times, respectively) was recorded in the roots of the three ecotypes, similar to what observed for the ion transporter genes.

Salt stress-induced accumulation of proline and free ABA

Free proline content in unstressed leaves resulted higher in ‘Domitiana’ than in the other two ecotypes both at 2 and 21 days (Table 2). Short exposure to NaCl significantly induced accumulation of this compatible osmolyte in ‘Canneto’ and ‘Cercola’ but not in ‘Domitiana’, whose proline levels in stressed leaves were nonetheless similar to those of the other two ecotypes. After prolonged NaCl treatment, proline accumulated significantly in all the ecotypes (Table 2).

Leaf expression analysis of the proline biosynthetic gene *P5CS*, coding for Delta-1-pyrroline-5-carboxylate synthetase, showed different transcription levels of this gene in the three ecotypes, that mostly paralleled those observed for the Na^+ homeostasis and transcriptional regulators genes. After 2 days, *P5CS* transcription was significantly lower in control leaves of ‘Cercola’ than in ‘Canneto’ and ‘Domitiana’ and was up-regulated after NaCl treatment only in ‘Canneto’ and ‘Cercola’ (Fig. 2). After 21 days, *P5CS* expression in control leaves was still significantly higher in ‘Domitiana’ than in ‘Canneto’ and ‘Cercola’, whose *P5CS* transcription reached the same levels of ‘Domitiana’ only in response to salt treatment.

Free ABA quantification in *A. donax* leaves revealed a strong accumulation of the hormone already after 2 days of NaCl treatment in the three ecotypes, though less so in ‘Canneto’ (73% increase compared to 212 and 232% of ‘Cercola’ and ‘Domitiana’, respectively), suggesting that osmotic stress was inducing stomata closure (Table 2). After 21 days, free ABA content in control leaves was similar between the three ecotypes, and accumulated in response to NaCl

Table 2 Proline and free ABA content in leaves of three *A. donax* ecotypes after 2 and 21 days of 150 mM NaCl treatment

Ecotypes	2 days		21 days	
	Control	150 mM NaCl	Control	150 mM NaCl
Proline (μmol g^{-1} FW)				
Canneto	2.09 ± 0.17 b	2.41 ± 0.36 a*	0.96 ± 0.14 b	2.68 ± 0.30 a*
Cercola	2.20 ± 0.25 ab	2.43 ± 0.20 a*	1.09 ± 0.23 b	1.62 ± 0.09 b*
Domitiana	2.36 ± 0.16 a	2.42 ± 0.19 a	1.75 ± 0.32 a	2.78 ± 0.54 a*
ABA (nmol g^{-1} FW)				
Canneto	3.63 ± 0.43 a	6.30 ± 1.16 a*	3.59 ± 0.35 a	4.43 ± 0.57 b
Cercola	2.11 ± 0.93 ab	6.59 ± 1.01 a*	3.40 ± 0.21 a	3.81 ± 0.79 b
Domitiana	1.39 ± 0.21 b	4.61 ± 0.46 b*	3.29 ± 0.44 a	6.20 ± 1.15 a*

Values are expressed as mean of three technical replicates from three biological samples ± SD. Within each treatment, means followed by the same letter are not statistically different (Duncan’s test; $P \leq 0.05$). Within each ecotype, differences between NaCl-treated and control plants indicated by * are significant (Duncan’s test; $P \leq 0.05$)

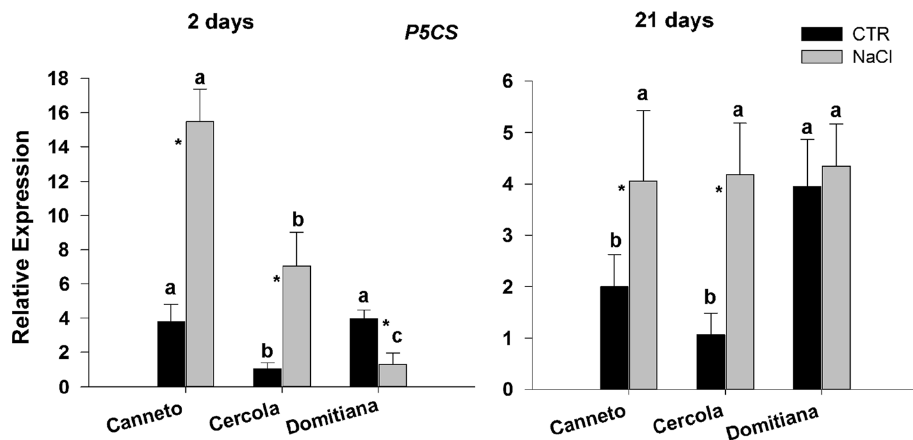

Fig. 2 Expression analysis of the proline biosynthetic gene *P5CS* in leaves of three *A. donax* ecotypes after 2 and 21 days of 150 mM NaCl treatment. qRT-PCR results are expressed as fold changes relative to the relevant unstressed control of the ecotype 'Cercola'. Values are the mean ± SD of three technical replicates from three biological samples. Within each treatment, means followed by the same letter are not statistically different (Duncan's test; $P \leq 0.05$). Within each ecotype, differences between NaCl-treated and control plants indicated by * are significant (Duncan's test; $P \leq 0.05$)

with 12, 23 and 88% increase in 'Cercola', 'Canneto' and 'Domitiana', respectively, though significant only in the latter (Table 2).

Ecotype response to NaCl-induced oxidative stress

Under control conditions, the levels of hydrophilic and lipophylic antioxidants were lower in leaves of 'Canneto' and 'Cercola' than in 'Domitiana'. However, after 21 days of salt treatment, a significant increase was observed for hydrophilic antioxidants in 'Canneto' and 'Domitiana' and for the lipophilic fraction in 'Canneto' and 'Cercola', in which it reached the same level found in 'Domitiana' (Table 3). Lipid peroxidation, measured as MDA content, was stimulated by salt stress after 21 days in all ecotypes (Table 3), but particularly in 'Domitiana', in which it increased by 65%.

Investigation of the redox state following saline stress in the three ecotypes of *A. donax* through expression analysis of ROS scavenging-related genes at day 21 of NaCl treatment (Fig. 3) revealed that the unstressed leaf transcription rates of glutathione reductase (*GR*) in 'Canneto' and superoxide dismutase (*SOD4*), catalase (*CAT1*) and *GR* in 'Domitiana' were higher than in 'Cercola' (Fig. 3). NaCl-triggered transcription was significant in leaves of 'Cercola' for *SOD4* and *GR*, while in 'Canneto' only for *GR*. In 'Domitiana', the only transcriptional change induced by NaCl was the down-regulation of *GR*. Similarly, to TFs and transporters genes, salt stress strongly induced the expression of *SOD4*, *CAT1* and *GR* in roots of all the ecotypes, with a higher than 100-fold increase compared to unstressed conditions (Fig. 3).

Changes of the leaf and root methylomes under salt stress

Cytosine methylation status at CCGG sequences in three *A. donax* ecotypes assayed by the MSAP technique demonstrated DNA methylation changes under salt stress in the leaf and root of all giant reed ecotypes (Table 4). A total of

Table 3 Comparison of antioxidant activity and lipid peroxidation in leaves of three *A. donax* ecotypes after 21 days of 150 mM NaCl treatment

	Hydrophylic antioxidants TEAC mmol Trolox kg^{-1} FW		Lypophylic antioxidants TEAC mmol Trolox kg^{-1}FW		MDA nmol g^{-1}FW	
Ecotypes	CT	150 NaCl	CT	150 NaCl	CT	150 mM NaCl
Canneto	0.049 ± 0.004 c	0.078 ± 0.01 a*	0.048 ± 0.003 b	0.072 ± 0.003 a*	1.350 ± 0.059 a	1.720 ± 0.055 b*
Cercola	0.065 ± 0.003 b	0.067 ± 0.005 b	0.051 ± 0.005 b	0.073 ± 0.005 a*	1.287 ± 0.034 a	1.796 ± 0.043 b*
Domitiana	0.073 ± 0.006 a	0.078 ± 0.006 a*	0.071 ± 0.003 a	0.076 ± 0.003 a	1.319 ± 0.069 a	2.181 ± 0.108 a*

The TEAC assay for hydrophilic and lipophilic antioxidant activity expressed in mmol Trolox kg^{-1} FW, and the thiobarbituric acid-reactive substances assay of total lipid peroxidation for MDA content, expressed in nmol g^{-1} FW, were carried out. Values are the means of three biological and technical replicates ± SD. Within each treatment, means followed by the same letter are not statistically different (Duncan's test; $P \leq 0.05$). Within each ecotype, differences between NaCl-treated and control plants indicated by * are significant (Duncan's test; $P \leq 0.05$)

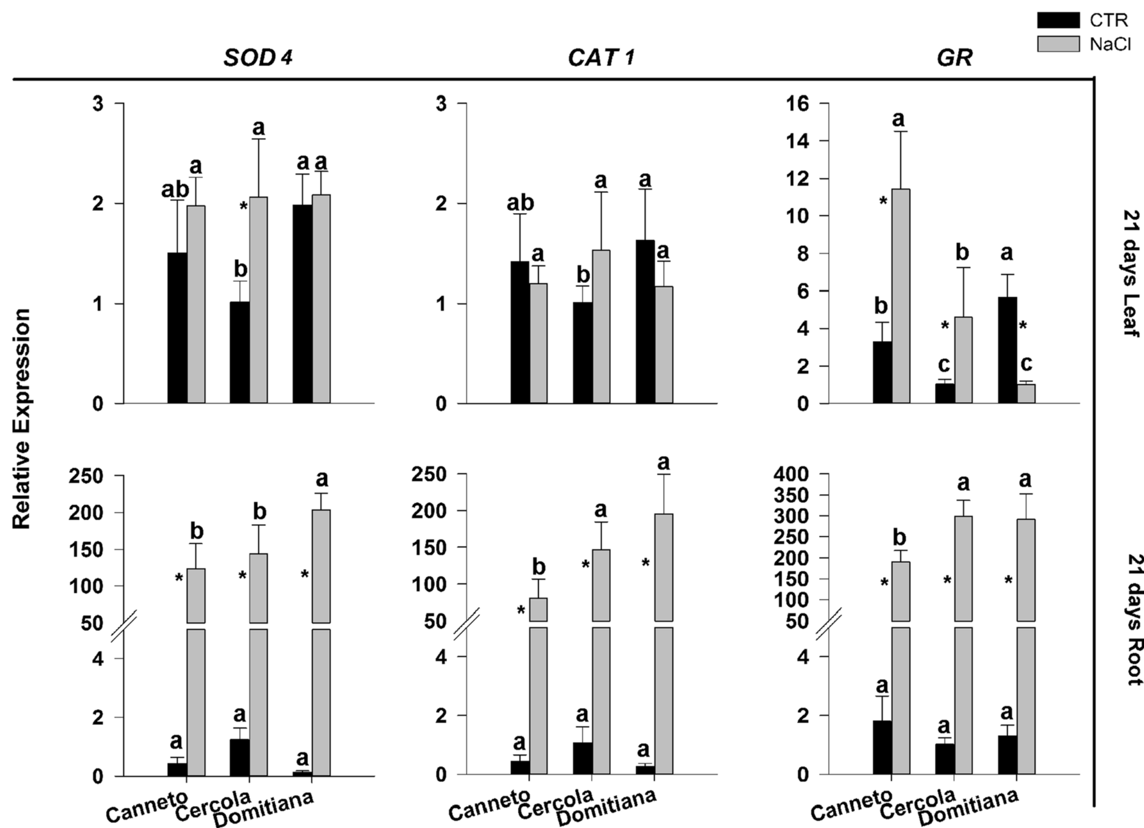

Fig. 3 Expression analysis of ROS detoxification genes in leaves and roots of three *A. donax* ecotypes after 21 days of 150 mM NaCl treatment. qRT-PCR results are expressed as fold changes relative to the relevant control of the ecotype 'Cercola'. Values are the mean ± SD of three technical replicates from three biological samples. Within each treatment, means followed by the same letter are not statistically different (Duncan's test; $P \le 0.05$). Within each ecotype, differences between NaCl-treated and control plants indicated by * are significant (Duncan's test; $P \le 0.05$)

5010 and 6846 scorable fragments were amplified in leaves and roots, respectively. Under control conditions, in the leaf, total methylated bands averaged 68.4% in 'Cercola', 70.5% in 'Canneto' and 72.2% in 'Domitiana', while in the root, the average ranged from 65.8% (in 'Domitiana') to 69.0% (in 'Cercola'). In this organ, salinity stress increased the percentage of total methylated sites in all ecotypes, whereas in the leaf, a genotype-specific response was observed. 'Cercola' showed a higher number of total methylated loci in treated leaf samples (avg 72.0%) compared to its controls, with an average increase of 3.6%. On the counterpart, in 'Domitiana', NaCl treatment decreased the total methylated sites (72.2% vs 69.8% in control and stressed leaves, respectively). Total methylation in leaves of 'Canneto' remained at a level similar to controls (avg 70.5% vs 70.4%) when exposed to salinity. As for the fully methylated bands, there was an evident increase in 'Cercola' samples under salinity in both tissues. Conversely, following NaCl treatment, the percentage of fully methylated sites respective to unstressed control decreased in 'Domitiana' leaf (avg 58.8% vs 54.2%) and increased in roots (56.1 vs 63.3%). In 'Canneto', salt-triggered changes of hemi-methylated loci resembled those observed in 'Domitiana' (Table 4). Regarding the hemi-methylated sites, salinity mainly increased them in all *A. donax* ecotypes both in the leaf and root. The only exceptions were roots of 'Domitiana' and 'Canneto', where the level of hemi-methylated bands remained either lower (avg 9.7 vs 7.4 in the former) or similar (avg 10.4 vs 10.6 in the latter). In the leaf, total and fully methylated bands increased in all three ecotype controls when comparing 2 vs 21 days of treatment, while an opposite trend was found in the root. Concerning the hemi-methylated bands, leaf and root samples showed a decrease from 2 to 21 days of treatment in all three ecotypes with percentages of 15.8 vs 8.6 and 10.9 vs 7.9 (avg), respectively. A general trend of higher level of fully and hemi-methylated bands was observed in the leaf and root under both control and high NaCl conditions in the three ecotypes. The fully methylated loci were always more than the hemi-methylated ones (Table 4).

The effects of short (2 days) versus prolonged (21 days) 150 mM NaCl treatment on CCGG sequences were evaluated. Overall, in the leaf, methylated bands decreased with

Table 4 DNA methylation in leaves and roots of three *A. donax* ecotypes after 2 and 21 days of 150 mM NaCl treatment as determined through MSAP analysis

Genotype	Treatment	Bands per pattern type, n.				Total methylated sites, n (%)	Fully methylated sites, n (%)	Hemi-methylated sites, n (%)
		I	II	III	IV			
Leaf								
Canneto	2-day control	138	61	67	120	282 (68.9)	212 (51.6)	70 (17.4)
	2-day NaCl	124	71	70	121	273 (68.7)	206 (50.5)	67 (18.1)
	21-day control	214	79	31	125	320 (72.2)	282 (65.3)	38 (6.9)
	21-day NaCl	173	89	62	125	309 (72.2)	247 (58.4)	62 (13.8)
Cercola	2-day control	140	84	46	116	270 (69.9)	224 (58.0)	46 (11.9)
	2-day NaCl	139	85	56	106	280 (72.5)	224 (58.0)	56 (14.5)
	21-day control	140	113	47	149	300 (66.8)	253 (56.3)	47 (10.5)
	21-day NaCl	163	101	57	128	321 (71.5)	264 (58.8)	57 (12.7)
Domitiana	2-day control	146	66	70	104	266 (73.1)	199 (54.9)	67 (18.1)
	2-day NaCl	163	43	67	113	265 (70.7)	195 (53.4)	70 (17.4)
	21-day control	222	60	38	129	324 (71.3)	293 (62.8)	31 (8.5)
	21-day NaCl	190	57	62	140	324 (68.8)	262 (55.0)	62 (13.8)
Roots								
Canneto	2-day control	261	117	73	182	443 (71.2)	373 (59.7)	70 (11.5)
	2-day NaCl	329	49	83	172	439 (72.8)	401 (59.7)	38 (13.1)
	21-day control	228	44	47	189	313 (62.8)	271 (53.5)	42 (9.3)
	21-day NaCl	234	80	41	153	367 (69.9)	322 (61.8)	45 (8.1)
Cercola	2-day control	330	65	63	175	458 (72.4)	395 (62.4)	63 (10.0)
	2-day NaCl	313	85	78	157	476 (75.2)	398 (62.9)	78 (12.3)
	21-day control	211	89	33	175	333 (65.6)	300 (59.1)	33 (6.5)
	2-day NaCl	330	65	63	175	458 (72.4)	395 (62.4)	63 (10.0)
Domitiana	2-day control	241	132	70	190	451 (70.0)	378 (58.9)	73 (11.1)
	2-day NaCl	324	77	38	194	461 (69.4)	378 (63.3)	83 (6.0)
	21-day control	206	65	42	195	319 (61.6)	272 (53.3)	47 (8.3)
	21-day NaCl	210	112	45	141	355 (72.2)	314 (63.4)	41 (8.9)

increased exposure time to excess NaCl, except for 'Domitiana' (at fully methylated sites), where the 21-day treatment affected the total number of methylated sites (355 in stressed root vs 319 in the control) more than the 2-day stress (461 vs 451). Similarly, in 'Canneto', the number of methylated sites was lower in stressed leaves (−3.3% of average variation). Again, in the root, the trend was towards a reduction of methylated bands from 2 to 21 days of NaCl treatment both in 'Cercola' and 'Canneto' (with the exceptions of fully methylated sites). However, in 'Domitiana', an opposite trend was observed.

The banding patterns between control and NaCl-treated tissues of the three *A. donax* genotypes were compared to find out all possible changes under salinity stress (Fig. 4, Table S3). The data obtained can be summarized as follows: 'Cercola' behaved as an early-responding ecotype, since it showed a higher amount of hypermethylation changes after 2 days compared to the other ecotypes. Regarding long-term stress (21 days) effects, 'Cercola' did not show considerable changes respect to 2-day samples. In addition, in the leaf, salt-induced methylation changes were substantially equally distributed between hyper- and hypo-methylation patterns, whereas in the root, hypermethylation was prevalent. Instead, 'Canneto' showed greater percentage of hypermethylation changes after 21 than after 2 days. Therefore, 'Canneto' behaved as a late-responding ecotype. Finally, 'Domitiana' showed an early response in the root (extensive hypomethylation at 2 days) and a late response in both tissues (with a wide hypermethylation at 21 days). To better estimate the predominant event between methylation and de-methylation, a parameter termed methylation ratio (MR) was calculated as the ratio between the hyper- and hypo-methylation frequencies in 'Cercola', 'Canneto' and 'Domitiana' NaCl-treated samples compared to their respective controls (Fig. 5). In 'Cercola', the hypermethylation changes were higher in the root than in the leaf at both 2 and 21 days (1.9 and 1.4 root; 1.0 and 0.9 leaf). In 'Canneto', a large increment of hypermethylation is registered in leaf and root at 21 days with MR values of 1.3 (root) and 1.7 (leaf). In 'Domitiana', we observed a higher hypermethylation at 21 than at 2 days in both tissues.

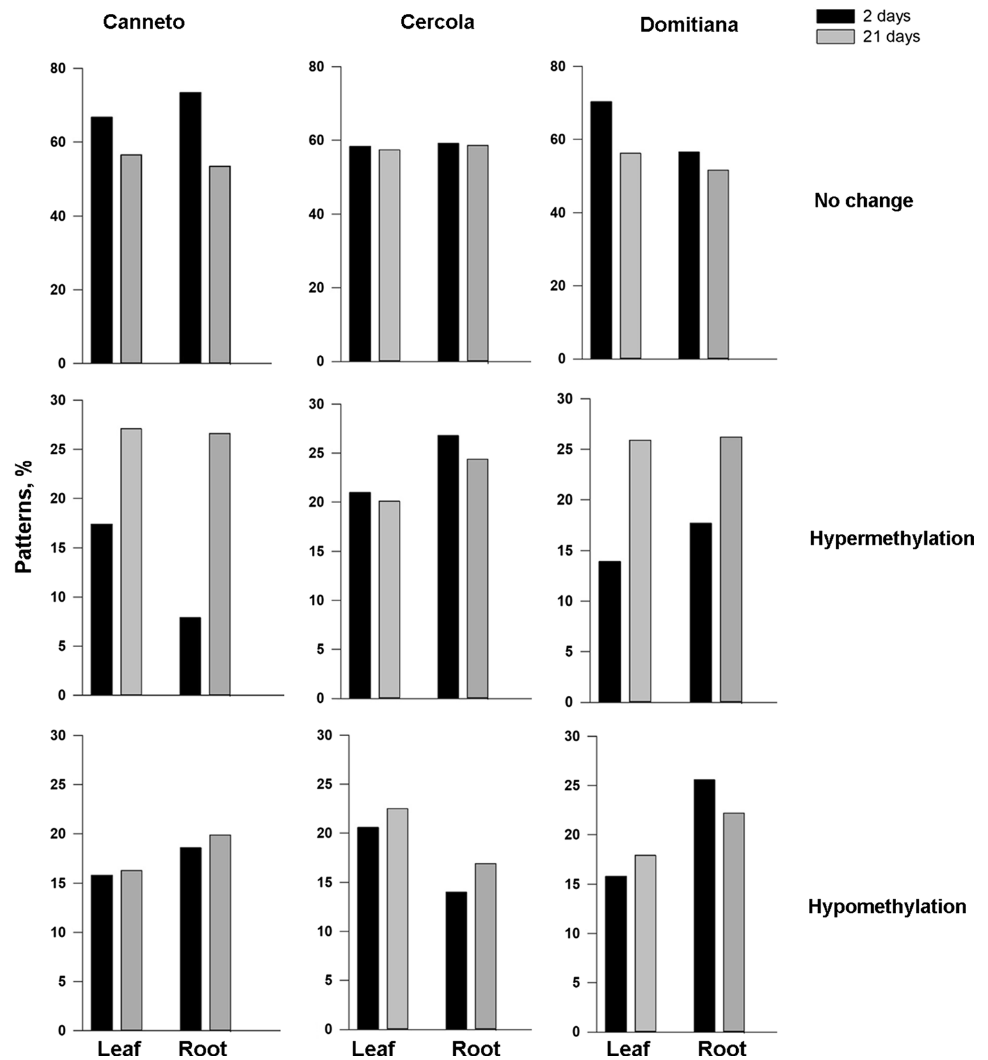

Fig. 4 DNA methylation patterns between control and salt-treated leaf and root of three *A. donax* ecotypes after 2 and 21 days of 150 mM NaCl exposure. The patterns, expressed as percentage of NaCl-treated over control samples, represent no change, hypomethylation, and hypermethylation events induced by salt stress

Discussion

Cultivation of the fast-growing invasive species giant reed (*A. donax* L.) is recently diffusing for ligno-cellulosic biomass production in warm and temperate climates. Due to its peculiar capability of maintaining high growth rates and yield in limiting environments, it appears particularly suitable in marginal lands and especially in saline soils, since it was classified as 'moderately salt tolerant' (Nackley and Kim 2015; Pompeiano et al. 2017). Despite the limited genetic variation due to its obligatory agamic reproduction system, recent studies have demonstrated that different varieties and ecotypes of *A. donax* can have distinct responses to abiotic stresses (Sánchez et al. 2015; De Stefano et al. 2017; Haworth et al. 2017a, b, c). These are of potential use for the selection of genotypes suitable for efficient and sustainable cultivation in specific environments. Therefore, we used three Italian ecotypes to investigate the early and late responses of leaf and root tissues to NaCl at the morpho-physiological, transcriptional, metabolic and DNA methylation level.

A. donax ecotypes differ in their morpho-physiological response to salinity

In our high-salinity experimental conditions (i.e. hydroponic growth in 150 mM NaCl, EC = 1.9 mS cm^{-1}), none of the *A. donax* ecotypes showed evident phenotypic symptoms of toxicity, such as chlorotic or necrotic leaves, even after prolonged exposure (21 days). Significant reduction in leaf number was detected only in 'Cercola', but was associated with decreased shoot height, thus indicating that, if accumulation of Na^{+} and Cl^{-} in older *A. donax* leaves was occurring (Pollastri et al. 2017), it did not induce premature leaf senescence in our ecotypes. Similarly, the response of an Italian *A. donax* ecotype to drought stress did not involve intense leaf shedding, opposite to what observed for a Moroccan ecotype, possibly reflecting different adaptive strategies to

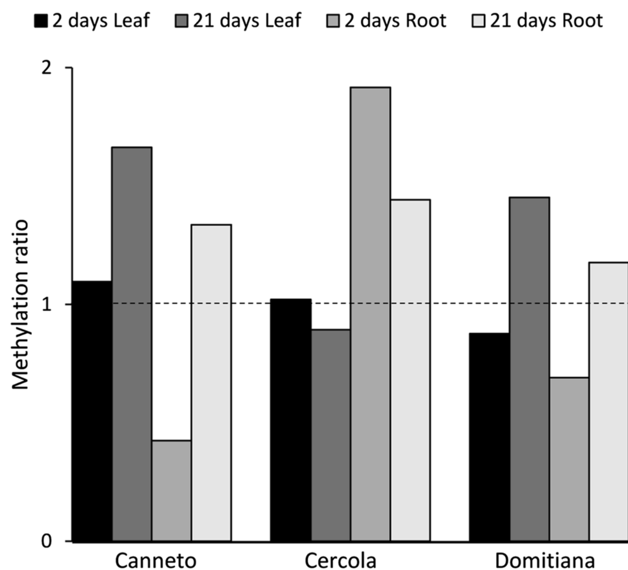

Fig. 5 DNA methylation ratio (MR) in leaves and roots of three *A. donax* ecotypes after 2 and 21 days of 150 mM NaCl treatment. MR values were calculated as the percent ratio of the number of MSAP bands revealing hypermethylation over the number of MSAP bands revealing hypomethylation, relative to unstressed leaves and roots

the respective environments (Haworth et al. 2017b). Elimination of excess toxic ions through shedding of older leaves seemed not taking place in our experimental conditions and ecotypes, whose salt tolerance might probably rely on the ability to exclude toxic ions and/or partially restrict Na^+ translocation and accumulation into the shoots. Indeed, retention of Na^+ and Cl^- in roots and rhizomes was reported for salt-treated *A. donax* (Pollastri et al. 2017; Pompeiano et al. 2017). SPAD values, which correlate with chlorophyll A content in *A. donax* (Spencer 2014), did not change or even slightly increased after salt treatment (Supplementary Fig. S3), in agreement with Sánchez et al. 2015 and Di Mola et al. 2018, indicating that the photosynthetic system was not damaged by excess NaCl. Accordingly, previous results demonstrated that the stress level used in this study did not affect chlorophyll content index or maximum quantum yield of PSII (F_v/F_m) in the three ecotypes (De Stefano et al. 2017). The observed increase in chlorophyll content could be due to increased leaf thickness (Longstreth and Nobel 1979) or to leaf area reduction or shrinkage (Tang and Boyer 2007), though this should not be the case since the fourth leaf width did not change significantly after the 21-day NaCl treatment (Table 1). Nevertheless, lack of stress damage cannot be unequivocally ruled out.

According to growth parameters, salinity negatively affected shoot and leaf biomass only in 'Cercola', while in 'Domitiana', it significantly stimulated root growth. The adaptive significance of root growth under salt stress remains elusive (Galvan-Ampudia and Testerink 2011). Tolerant Arabidopsis ecotypes reduce main root growth under severe stress to limit excess sodium internalization, whereas in other species, enhanced root growth to avoid locally high salt concentrations is associated to stress tolerance (Bernstein and Kafkafi 2002). Therefore, 'Domitiana' may have developed a mechanism of salt avoidance through a root system able to take up water from deep soil layers, as reported for other species (Munns and Tester 2008; Yoshimura 2008; Julkowska and Testerink 2015). From these results, we considered that, in terms of plant productivity (aerial biomass growth), the salt tolerance of the three ecotypes is 'Cercola' < 'Canneto' < 'Domitiana', consistently to what recently reported by (De Stefano et al. 2017).

Transcriptional and metabolic dynamics under short and prolonged salt excess in *A. donax* are ecotype and tissue dependent

Limitation of Na^+ and Cl^- accumulation in the more susceptible tissues, such as photosynthetic leaves, and detoxification of excess ions in cells and tissues represent key mechanisms of salt tolerance. Therefore, the study of genes involved in the regulation of Na^+ flux as well as salt sensing and signalling is crucial for understanding survival and growth of *A. donax* in saline soils, especially in light of our results indicating that other mechanisms, such as compartmentalisation in older leaves and elimination through leaf shedding, do not take place in the tested experimental conditions.

In giant reed, the transcriptional behaviour of the plasma membrane *SOS1* and the tonoplast *NHX1*, major players of Na^+ exclusion back to the soil and compartmentalization in the vacuole, respectively, as well as of *HKT1*, an essential contributor to K^+/Na^+ homeostasis and leaf protection from ion toxicity through unloading of Na^+ from the xylem sap (Almeida et al. 2017) demonstrated ecotype-dependent regulation. Salt-induced over-expression of ion transporter genes in 'Cercola' after 2 days may indicate that, in response to substrate salinity, 'Cercola' regulates ion homeostasis more rapidly than the other two ecotypes, maintaining this trend of regulation after prolonged exposure to NaCl. However, either the putative reduction of ionic stress is not sufficient to protect this ecotype from shoot growth inhibition or shoot biomass reduction is a further mechanism to limit toxic Na^+ and Cl^- accumulation by decreasing H_2O consumption and total evapotranspiration. On the contrary, unchanged or reduced expression suggests that support of plant growth under high salinity in the other two ecotypes may be associated to an already higher expression of ion transporter genes in the absence of stress. Albeit the divergence between ecotypes in the NaCl response may not be associated to genetic variation, the high constitutive level of

expression of a set of stress-responsive genes might reflect the adaptation of these ecotypes to the original growing environments. This observation is in agreement with previous studies, which reported that *A. donax* ecotypes respond in a very different manner to drought stress, depending on their capability to adapt to environmental constraints (Ahrar et al. 2015; Haworth et al. 2017a, b, c).

As far as key TFs contributing to salt tolerance, we evaluated *WRKY53* and *DREB2A*, since they have a synergic role in the improvement of salt stress tolerance (Sakuma et al. 2006; Mallikarjuna et al. 2011; Allu et al. 2014; Van Eck et al. 2014; Song et al. 2016) and have been demonstrated to transduce a wide range of stress-responsive signals (Mallikarjuna et al. 2011) in many plant species. The transcriptional scenario observed for *WRKY53* and *DREB2A* TFs mirrored that of the ion transporters. NaCl-triggered *WRKY53* transcription was demonstrated in *A. thaliana* (Sun and Yu 2015), similar to the trend observed in the *A. donax* ecotypes 'Canneto' and 'Cercola', except for 'Canneto' roots after short NaCl exposure (Fig. 1). *WRKY53* was suggested to be implicated in contrasting NaCl-induced leaf senescence in rice (Khan et al. 2017) and may contribute to the absence of leaf yellowing and decay in our experiments, possibly through activation of the antioxidant machinery. Accordingly, antioxidant accumulation and *SOD4* and *GR* over-expression were more intense in the above two ecotypes (Table 3 and Fig. 3). Indeed, *AtWRKY53* could contribute to alleviate oxidative damage, as suggested by decreased H_2O_2 accumulation during osmotic stress in over-expressing lines (Sun and Yu 2015). The already elevated levels of *WRKY53* as well as of antioxidants and antioxidant transcripts in 'Domitiana' unstressed leaves (Table 3 and Fig. 3) might allow protection from NaCl-induced oxidative stress and explain the ability of 'Domitiana' to tolerate higher levels of lipid peroxidation without compromising plant growth. Rice *DREB2A* over-expression in soybean up-regulated proline biosynthesis and sugar accumulation, thus showing the involvement of this TF in many stress-related pathways (Zhang et al. 2013). The early (2 days) up-regulation of *DREB2A* induced by NaCl in 'Canneto' and 'Cercola' leaves does not unequivocally explain the contribution of this TF to salt tolerance in *A. donax*. Nevertheless, it is tempting to speculate an interaction with other metabolic partners leading to an increase in leaf proline of 'Canneto' and 'Cercola' after NaCl treatment, the former reaching the same levels as 'Domitiana'. Proline accumulation in response to NaCl appears to be complex in *A. donax*, since in 'Domitiana' it does not strictly correlate to the *P5CS* gene expression. This result suggests that, as hypothesised by Pollastri et al. (2017), de novo synthesis via glutamate or ornithine pathway through the P5CS enzyme might not be the only regulation of proline levels under salt conditions, since reduction of catabolic processes may also contribute to adaptive mechanisms, as demonstrated in *Medicago sativa* (Miller et al. 2005).

Abscisic acid has a pivotal role in plant adaption to the osmotic component of salinity stress, along with osmolytes, and in the regulation of stomatal closure under long-term adaptation to osmotic stress (Maggio et al. 2007). Therefore, while 'Cercola' reacts to salinity with a stronger and earlier activation of the ion homeostasis machinery, the limited effect of salinity on plant biomass in 'Canneto' and 'Domitiana' may be due, at least in part, to the observed higher increase in free ABA levels following prolonged salt stress, and hence to the higher ability of these two ecotypes to preserve cell turgor and expansion, thus allowing plant growth. However, stomatal closure also limits gas exchange and photosynthesis. Indeed, using the same experimental conditions, we previously demonstrated significant NaCl-dependent inhibition of stomatal conductance and assimilation rate in all the three ecotypes, though in the absence of permanent damage to the photosynthetic apparatus (De Stefano et al. 2017). This suggests dissipation of excess energy through photo-oxidation and production of free radicals in all the three ecotypes, as also indicated by increased leaf accumulation of MDA, a by-product of lipid peroxidation that estimates membrane damage by oxidative stress (Dhindsa et al. 1981). Consequently, the level of leaf antioxidants, hydrophilic and/or lipophilic, increased in all ecotypes, but especially in 'Canneto', with 'Domitiana' having the highest levels both in control and NaCl-treated leaves. This response corresponds to increased leaf transcription of *GR* in 'Canneto' and 'Cercola' and *SOD4* in 'Cercola'. 'Domitiana', which accumulated antioxidants already in control conditions, showed decreased or not significantly different leaf expression of antioxidant genes and, consistently, of *WRKY53*, which is involved in regulation of the antioxidant machinery (Sun and Yu 2015).

Ion transporters and TFs genes, as well as antioxidant genes, were over-expressed at very high levels in roots exposed to prolonged NaCl salinity in all the three ecotypes. Overall, our transcriptional analysis detected the strongest transcriptional response to salinity in late roots, indicating that the stress level in the roots, which are directly in contact with the stressing agent, is high enough at this time point to require the recruitment of all the possible tolerance strategies in all the three ecotypes. Accordingly, higher transcriptional responsivity of roots compared to leaves was also demonstrated in *A. donax* under water stress (Fu et al. 2016). The root response to 2 days of NaCl treatment is indicative of the different stress tolerance levels of the three ecotypes. 'Cercola' is more susceptible and shows over-expression of both TFs, which may hence activate drought-specific transcription control, as well as of the genes responsible for Na^+ exclusion and K^+ homeostasis, to cope with the stress.

Contrastingly, 'Canneto' and 'Domitiana' appear to have higher constitutive defences that make them tolerate the 2-day NaCl stress intensity without activating transcription of the above genes.

A. donax ecotypes adjust DNA methylation levels differently

To verify whether differences between the ecotypes may extend to the methylome, we profiled the methylation status of the three ecotypes. First, we observed that the amount of DNA methylation varied in all ecotypes from 2 to 21 days of treatment, confirming that plant exposition to salt can induce a reorganization at the cellular level in epigenetic and hence transcriptional terms. Variations of global DNA methylation levels have also been reported in several plant species both for abiotic and biotic constrains (Zhang et al. 2018; De Palma et al. 2019; Ferreira et al. 2019), emphasizing the role of differential methylome flexibility as an important player in stress response. Second, we found that DNA methylation varied between roots and leaves, especially at 2 days after treatment in 'Canneto' and 'Cercola'. This emphasizes that salt-induced DNA methylation changes can be tissue specific, consistently with some authors who argued that tissue-specific biological functions should imply a distinct gene regulation, eventually involving differential DNA methylation (Aceituno et al. 2008). However, how these tissue-specific changes in DNA methylation patterns are triggered by high salinity remains to be elucidated for facilitating breeding programs. Third, we observed that our ecotypes differed in the hyper- and hypo-methylation patterns when grown under high NaCl conditions. In particular, the evident salt-triggered methylation observed in 'Canneto' and 'Domitiana' (in both leaves and roots) reflects what found in several plant species in response to environmental cues, such as cold, heavy metals and salt (Lízal and Relichová 2001; Aina et al. 2004; Choi and Sano 2007). In contrast, in 'Cercola', the length of salinity treatment affected the methylation levels, triggering an overall depletion of mC sites in both root and leaf. These findings agree with other studies demonstrating that closely related genotypes may possess some methylome flexibility. For example, rice cultivars with different salinity tolerance capacities exhibited different DNA methylation patterns in response to NaCl (Ferreira et al. 2015; Garg et al. 2015; Wang et al. 2015). Similarly, salinity inconsistently affected the level of DNA methylation in two different canola genotypes (Marconi et al. 2013). Overall, we found that all giant reed ecotypes were able to rapidly change DNA methylation (either reducing or increasing) upon stress imposition.

Conclusions

This study highlighted ecotype- and tissue-dependent variation in key mechanisms of salt response of *A. donax* to short- and long-term excess NaCl. The results concur in indicating that, among the ecotypes, the 'Cercola' reaction to salt excess was more rapid and intense than the other two ecotypes, though this apparently occurs at the expenses of plant growth. On the contrary, 'Domitiana' and, to a lesser extent, 'Canneto' may have constitutively elevated defences against high NaCl and, following NaCl treatment, may redirect energy to other tolerance mechanisms, such as stress avoidance through enlarged soil exploration by the root. DNA methylation analyses suggested a link between the plant performance under high salinity and the DNA methylation plasticity, especially in 'Domitiana'. Our findings demonstrated that tolerant ecotypes possess a wide reservoir of molecular and metabolic resources to face salinity already in the absence of stress, possibly due to adaptation to their original growing environments. This may help in mitigating salinity damage in the early phase of stress, thus allowing the use of a "power saving mode" strategy.

Though the complexity of the salinity tolerance trait in *A. donax* will require further investigations to elucidate the relative contribution of different mechanisms to adaptation, our results could be useful for design breeding programs aimed at obtaining genotypes more suitable for efficient and sustainable ligno-cellulosic biomass production in limiting environments.

Author contribution statement TD and MT designed research; TD, RDS, MDP, and EC performed morpho-physiological, transcriptional and metabolic analyses; CV and RA designed and performed MSAP profiling and analysis; TD and MT interpreted data and wrote the paper. All authors read and approved the manuscript.

Acknowledgements We are thankful to Prof. G. Caruso (Università di Napoli Federico II, Italy) for valuable help with statistical analysis. Assistance of Mr. G. Guarino and Mr. R. Nocerino (CNR-IBBR, Portici, Italy) with artworks and plant growth, respectively, is gratefully acknowledged. This work was partially supported by a research grant from the Italian Ministry of Education, University and Research, Project BioPoliS (PON03PE_00107_1).

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