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Overexpression of the Arginine Decarboxylase Gene Improves Tolerance to Salt Stress in *Lotus tenuis* Plants

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Abstract To analyse the putative effects of putrescine on the plant response to salinity, micropropagated *Lotus tenuis* plants from wild-type and transgenic lines harbouring the *pRD29A::oat arginine decarboxylase* (ADC, EC 4.1.1.19) construct were subjected to a gradual increase in salinity that was applied gradually by means of sodium chloride irrigation (from 0 to 0.3 mol L^{-1}) every 5 days up to the maximum concentration. At the end of the experiment, the transgenic lines were healthier than the wild-type plants and displayed a smaller reduction in shoot biomass and a slight increase in root growth in response to stress. The overexpression of *ADC* increased osmotic adjustment (5.8-fold) via the release of proline. The salinity treatment doubled the potassium uptake by roots from transgenic ADC stressed plants with a concomitant decrease in the accumulation of sodium, balancing the Na^+/K^+ ratio. Analysis of gene expression, enzymatic activities and hormone metabolism suggests a crosstalk between polyamines and abscisic acid in response to salinity via modulation of the abscisic acid biosynthesis-related enzyme 9-*cis*-epoxycarotenoid dioxygenase (EC 1.13.11.51) at the transcriptional level.

Keywords Putrescine · Abscisic acid · NCED · Salt tolerance

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Introduction

Plant growth and productivity are often dramatically reduced by abiotic stresses. Among them, soil salinity has emerged as the most significant agricultural problem dropping crop yields worldwide (Flowers 2004). It is widely known that salinity occurs through natural or human-induced processes that result in the accumulation of dissolved salts in the soil water; high salinity inhibits plant growth and reduces the productivity of crops (Roy and others 2014). Growth is reduced by salinity via several distinct processes, including a reduction in water availability during the first phase and an accumulation of salt during the second phase of plant response. Biochemical and molecular mechanisms of salt tolerance in plants include production of suitable osmolytes, ion compartmentalization and induction of enzymes that produce antioxidants and phytohormones (Munns and Tester 2008). Under these circumstances, the accumulation of different organic compounds, including polyamines (PA), and the expression of several genes have been described as part of the signaling and defence systems of plants against salinity stress (Zhou and others 2007).

Polyamines, including putrescine (Put), spermidine (Spd) and spermine (Spm), are low molecular weight compounds with aliphatic nitrogen structures that are found in almost all organisms, ranging from bacteria to animals and plants (Marco and others 2011). PA play a key role in plant development and stress protection (Hussain and others 2011; Minocha and others 2014), including *Lotus* species (Calzadilla and others 2016). The diamine Put is synthetized by either arginine decarboxylase (ADC) or ornithine decarboxylase (ODC). Additionally, Spd and Spm are synthetized by spermidine synthase and spermine synthase via the addition of aminopropyl groups to Put and Spd,

respectively. Furthermore, Put, Spd and Spm are degraded by copper amine oxidase and polyamine oxidase, producing aminoaldehydes, ammonia and H_2O_2 (Calzadilla and others 2014).

The expression or overexpression of several genes encoding enzymes involved in the biosynthetic and metabolic pathways of PA promote the expression of functional and regulatory genes involved in stress tolerance (Gill and Tuteja 2010; Minocha and others 2014; Zarza and others 2016). PA maintained cell stability and ion homeostasis (Sharma and others 2011; Zhang and others 2014) by decreasing the uptake of Na^+ and increasing the K^+, Ca^{2+} and Mg^{2+} concentrations (Tang and Newton 2005). PA play a protective role as channel blockers (Zepeda-Jazo and others 2011) and may act as cofactors in the activation of H^+ pumps (Janicka Russak and others 2010). In addition, PA serve as compatible solutes (Minocha and others 2014), protect the photosynthetic apparatus (Gill and Tuteja 2010; Zhang and others 2014), control the movement of stomata (Zepeda-Jazo and others 2008), and regulate shoot and root growth (Wang and others 2011). They may also act as free radical scavengers and antioxidant enzyme activators, but they may also be a source of H_2O_2 (Tiburcio and others 2014) involved in signal transduction and in interactions with other hormones (Hussain and others 2011; Minocha and others 2014). To understand how PA levels are involved in the response of *L. tenuis* to salt stress, we previously obtained transgenic plants that overexpress the oat *ADC* gene under the control of the *Arabidopsis thaliana* stress-inducible promoter *pRD29A* (Espasandin and others 2010). The objectives of this study were to investigate the effect of the overexpression of the oat *ADC* gene on the tolerance response of *L. tenuis* to salt stress and polyamine metabolism and to examine its crosstalk with the abscisic acid (ABA) signaling process. The relationship between PA and ionic homeostasis is also discussed.

Materials and Methods

Plant Cultivation and Treatments

Assays were performed with *Lotus tenuis* cv INTA PAMPA. Micropropagated plants of similar age and size from wild-type (wt) and a transgenic line (Lt19) harbouring a single insertion of *pRD29A::oatADC* (Espasandin and others 2010) were cultured in 2 L pots filled with a mixture of sterile soil and perlite (1/1 w/w) with 0.5 g of controlled release micro-fertilizer (Osmocote; N/P/K, 9/45/15; 180 day release). The plants were placed in a growth room for 6–8 weeks under a day/night air temperature of 25–27/20–22 °C, a substrate temperature of 22–25 °C and a 14/10 h light/dark photoperiod (345 µmol m^{-2} s^{-1} PPFD, in the wavelength range of 400–700 nm provided by fluorescent lamps). We selected this transgenic line using the results obtained in previous studies as criteria within the different lines generated in one single transgenic event (Espasandin and others 2014) and considering their relative inductions of transgene expressions and the subsequent activity of decarboxylase.

Six-month-old wt and transgenic plants at the vegetative stage were subjected to a controlled experiment in which NaCl was applied gradually from 0 to 0.3 mol L^{-1} by means of irrigation every 5 days up to the maximum concentration (increased concentration of 0.05 mol L^{-1} each time). The samples were collected after 35 days of the experiment when the electrical conductivity of the soil solution reached 30.3 ± 11.1 dS m^{-1}. This approach helped to circumvent artificial changes in gene expression and physiological responses, which may be induced by a shock treatment (Shavrukov 2013). Non-stressed (control) plants were also included. Finally, to detect if there is a crosstalk relationship between Put and ABA and to validate the response of transgenic lines, a new experiment was performed by exogenous supply of putrescine dihydrochloride 0.62 mmol L^{-1} (98% purity), (±) *cis, trans*-ABA 0.38 mmol L^{-1} (99% purity) or distilled water (control), until incipient runoff (approximately 5 mL of aqueous solution per plant). All chemicals used were from Sigma-Aldrich (St. Louis, MO, USA). The solutions included Triton® X-100 (0.1%) as the surfactant, and a minimum amount of ethanol was used to dissolve the ABA. Spray treatments were repeated once a week until the final concentration of 0.3 mol L^{-1} NaCl was reached. The electrical conductivity of the soil solution was determined following the protocol described by Rhoades and others (1989).

Plant Harvest and Analysis

After 35 days of salt stress treatment, three samples of shoot tissues (stems and leaves) were harvested from each plant to measure osmotic potential (Ψ_π^{100}) and three samples were harvested to measure the relative water content (RWC). The Ψ_π^{100} was measured with a C-52 thermocouple and the leaf osmotic adjustment ($\Delta\psi_\pi^{100}$) under salt stress was calculated as the difference between non-stressed and stressed stages. The RWC was determined using the following formula: $\text{RWC}\ (\%) = [(\text{FW} - \text{DW})/(\text{TW} - \text{DW})] \times 100$; where: FW, DW and TW; fresh, dry and turgid weight, respectively. Net photosynthesis rate (P_N), transpiration (E) and stomatal conductance (g_s) were measured at light saturation (1500 µmol m^{-2} s^{-1} PPFD, LED light) with a portable photosynthesis system (LI-6400, LiCor, NE, USA). Measurements were taken at mid-day using the third fully expanded apical leaves. Sample data were calculated from three biological replicates.

The leaf concentrations of chlorophyll (Chl) a and b were determined using fresh material after the extraction of pigments in cold acetone. The pigment concentration was calculated according to Vernon (1960). The proline concentration was spectrophotometrically determined using the ninhydrin reaction (Magné and Larher 1992). Membrane damage was probed with an electrolyte leakage test as described by Verslues and others (2006). Malondialdehyde (MDA) was measured spectrophotometrically using the common method referred to as the thiobarbituric acid-reactive substances (TBARS) assay (Hodges and others 1999). The concentrations of Na^+ and K^+ were quantified by flame photometry (Zeltec, ZF-250, Dicrom Ing., Argentina). For the determination of each parameter, three samples per plant were collected ($n=9$). At the end of the experiment, the number of shoots, root and shoot dry weight were determined for each plant.

Enzyme Activity Assays

Arginine decarboxylase (ADC, EC 4.1.1.19) was extracted by homogenizing the shoot (stem and leaves) tissues in 100 mmol L^{-1} phosphate buffer (pH 7.5) containing 0.5 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA), 10 mmol L^{-1} dithiothreitol, 1 mmol L^{-1} pyridoxal phosphate and 20 mmol L^{-1} sodium ascorbate. The crude extracts were clarified by centrifugation at 10,000×*g* for 10 min. All procedures were carried out at 4 °C. The protein concentrations in the supernatants were determined using the standard Bradford assay (Bradford 1976). Enzyme activities were determined by mixing the extract with the substrate solution in a glass tube fitted with a rubber stopper and a filter paper disc soaked in 2 mol L^{-1} KOH. The substrate solutions for the determination of the ADC activity contained 1 mmol L^{-1} non-radioactive substrate amended with 50 nCi mL^{-1} L-[$^{14}C_1$]arginine. After 1 h of incubation at 37 °C, the reactions were stopped, and $^{14}CO_2$ was released by adding 200 µL of 10% (v/v) perchloric acid. Following a 1 h distillation of $^{14}CO_2$ at 37 °C, the filter paper was immersed in 200 µL of scintillation cocktail (4 g Omnifluor in toluene), and the radioactivity was determined using a Beckman LS 5000 TD scintillation counter (Beckman Coulter, Brea, CA).

Extraction and Determination of Polyamines

To determine the levels of free Put, Spd and Spm in leaf extracts, a sample of 0.300 gr of FW was ground in liquid nitrogen, extracted in 1 mL 5% (v/v) perchloric acid and incubated overnight at 4 °C. After centrifugation at 10,000×*g* for 15 min, 5 µL of 10 mmol L^{-1} 1.7-heptanediamine (ICN Biomedicals, Costa Mesa, CA) was added as an internal standard to 200 µL aliquots of leaf extracts; this was followed by the addition of 200 µL saturated Na_2CO_3 and 400 µL dansyl chloride. The mixture was then incubated overnight in the dark at 70 °C. The reaction was stopped by adding 100 µL of proline and dansylated amine was extracted in 500 µL of toluene. The organic phase was vacuum evaporated and the dansylated PA were dissolved in 200 µL of acetonitrile and analysed by reversed phase high-performance liquid chromatography, as described previously by Garriz and others (2004). The amount of soluble-conjugated PA was measured after the liberation of PA by acid hydrolysis, and 200 µL of the perchloric acid extract was hydrolysed with the same volume of 12 mol L^{-1} HCl during 18 h of incubation at 110 °C. The hydrolysed product was dried and dissolved in 200 µL of 5% (v/v) perchloric acid, and the level of PA was measured as described above (Rodríguez-Kessler and others 2008).

Quantitative Real-Time PCR Expression Analysis

Total RNA was extracted with the SV Total RNA Isolation System (Promega) according to the manufacturer's instructions. A total of 1 µg of RNA was reverse transcribed to cDNA with random hexamers using SuperScript™ III RT (Invitrogen). The qPCR reactions were prepared with the SYBR Green PCR Master Mix (Applied Biosystems, California, USA). The reaction mix for each well was 2.5 µL of cDNA, 10 µL of specific primers mix and 12.5 µL of the Master Mix in a final volume of 25 µL. The specific oat *ADC* primer sequences were 5′-AGT TAC GAC GTG AAA CAG GAT ATC A-3′ (forward) and 5′-CCA CCA TTT CCC ACA CCT TA-3′ (reverse), and for *LjNCED3*, they were 5′-ATA GGG AAC CCT GGA TGG AA -3′ (forward) and 5′-GAG AAG GAA TGG AAA TCT GAG C-3′ (reverse). *Lotus corniculatus β-tubulin* was amplified as a reference gene to normalize expression. The *β-tubulin* primer sequences were 5′-GTG GAG TGG ATC CCC AAC AA-3′ (forward) and 5′-AAA GCC TTC CTC CTG AAC ATG G-3′ (reverse). Three biological replicates were amplified in triplicate. The reaction mixtures were incubated at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 1 min using the 7500 Real-Time PCR System (Applied Biosystems). The results were processed using the 7500 Software v2.0.1 (Applied Biosystems) to determine the relative expression level and the significance of the measurements.

Statistical Analysis

The data were statistically analysed, and the means were compared by performing a t test calculated by the statistical software GraphPad version 7.0 (San Diego, CA, USA). The data are presented as the means ($n=3$) and the standard

error of the mean (SEM). The experiments were repeated three times.

Results

Effect of Salt Stress on Oat ADC Expression, Enzymatic Activity and Hormone Metabolism

The Lt19 line showed a 31-fold increase in *ADC* expression (Fig. 1a), increasing the enzymatic activity by 3.2-fold (Fig. 1b) compared with the non-stressed control of the same genotype, whereas the stressed wt plants showed a 2.1-fold increase relative to the control. No significant differences in ADC activity between non-stressed and stressed wt plants were observed. However, the accumulation of free Put in the leaves of the stressed Lt19 line diminished by a factor of 4.4 (Fig. 1c), and this result may be related to a higher consumption of Put under such circumstances. Additionally, the level of Spd (which is synthetized from Put) of the Lt19 line diminished significantly in response to a severe stress, whereas the concentration of Spm remained unchanged (Fig. 2). Put content in the conjugated fraction significantly decreased by 3.4-fold ($P<0.05$) in the leaves of the wt genotype under salt stress conditions (Fig. 1c). Considering that the expression of 9-cis-epoxycarotenoid dioxygenase (NCED, EC 1.13.11.51), which is a key enzyme that regulates ABA biosynthesis under stress, can be controlled by Put, we determined the relative expression of the *NCED* gene under salt stress and noticed that the relative expression of *NCED* was higher in the stressed Lt19 line than in wt plants, displaying a 10-fold greater expression (Fig. 3). Moreover, we observed a positive relationship between the expression of the oat *ADC* and *NCED* genes ($P=0.00009$).

When the wt plants were sprayed weekly with distilled water, Put or ABA, the concentration of free Put in the leaves of stressed plants increased in response

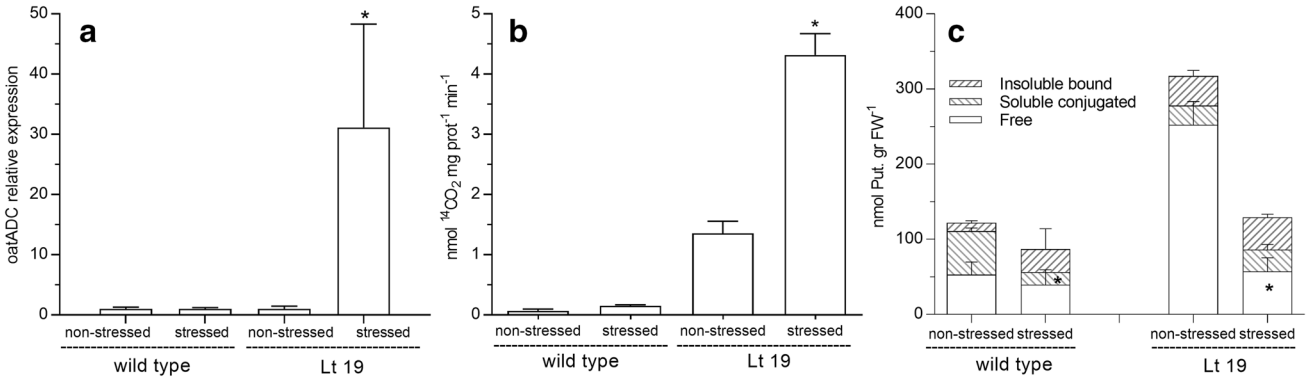

Fig. 1 *Oat ADC* relative expression (**a**), ADC activity (**b**) and putrescine content (**c**) in the pRD29A: oat ADC Lt19 line and in wt plants under 35 days of salt stress. The *values* represent the mean ($n=3$) ± SEM. *Asterisk* indicates significant differences with respect to the non-stressed control treatment (*t* test)

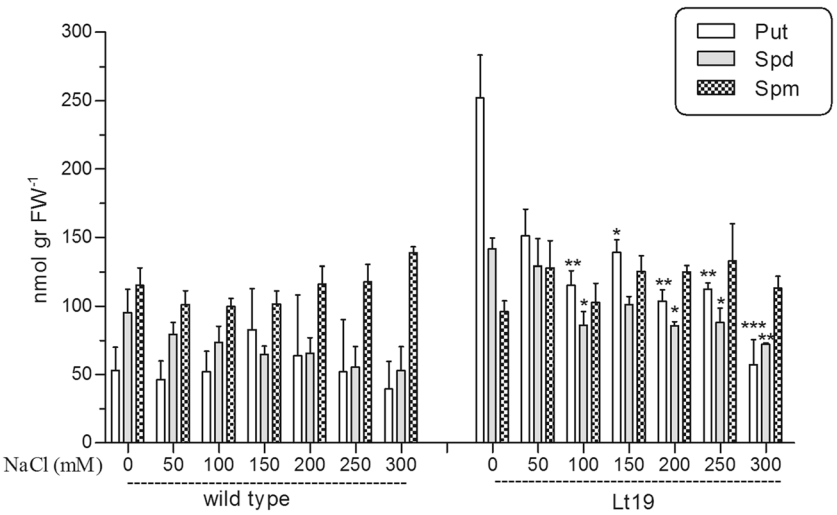

Fig. 2 The effect of different levels of salinity on the content of free polyamines of wt and transgenic plants. The *values* represent the mean ($n=3$) ± SEM. *Asterisks* indicate significant differences (*$P<0.05$, **$P<0.01$, ***$P<0.001$) with respect to the non-stress control treatment

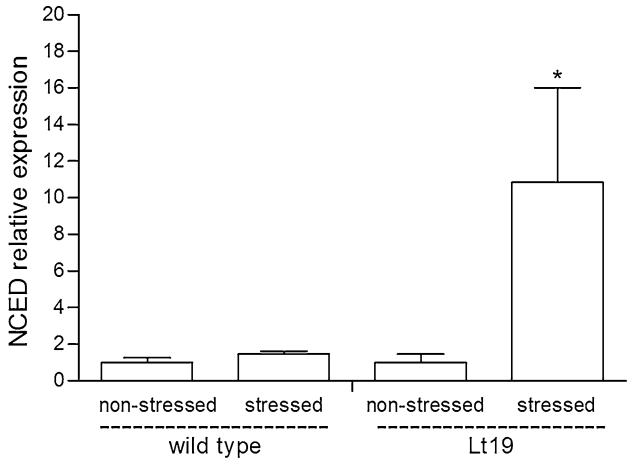

Fig. 3 Relative gene expression of NCED in *pRD29A:oatADC* Lt19 line and wild-type salt-stressed plants. The *values* represent the mean (n = 3) ± SEM. *Asterisk* indicates significant differences with respect to the non-stressed control treatment (*t* test)

to exogenous ABA (Fig. 4a). The level of Spd diminished due to the stress per se (distilled water) or the foliar application of Put; whereas the level of free Spm increased at the expense of Spd by the exogenous supply of Put. In contrast, the endogenous contents of free Put, Spd and Spm from the shoots of stressed Lt19 plants were not affected by the application of ABA (Fig. 4b). The levels of Put and Spd were diminished by the application of distilled water and Put; whereas the endogenous content of Spm remained unchanged.

Effect of Salt Stress on Gas Exchange, Water Content and Membrane Stability

The relative water content of the leaves slightly decreased in response to salinity (Table 1) and correlated with a sharp decline in stomatal conductance and transpiration. Concurrently, the leaf osmotic potential from the transgenic line decreased significantly and displayed an osmotic adjustment that was close to −2 MPa ($P < 0.01$). Under non-stress conditions, Lt19 plants accumulated nine times less proline in their leaves than did the wt plants. In both genotypes, salinity caused significant increases in the osmolyte content. During the stress period, the amount of proline in the leaves of Lt19 increased significantly ($P < 0.001$) relative to that in the unstressed plants (by a factor of 24). Under similar experimental conditions, the proline levels in the leaves of wt plants increased by a factor of 3.6 in relation to the corresponding controls ($P < 0.05$). Further, a significant increase in the MDA concentration was detected in the Lt19 line under salt stress but no damage at the level of cell membranes was detected (Table 1). The photosynthetic rate of both genotypes decreased in correspondence with the reduction in stomatal conductance. The chlorophyll content (Chl a, b and a + b) remained almost unchanged in both genotypes.

Effect of Salt Stress on the Na^+/K^+ Ratio

Both genotypes showed similar accumulation of Na^+ in stems and leaves under 0.3 mol L^{-1} NaCl (Table 2). In roots, the concentration of Na^+ was higher in wt than in Lt19 plants subjected to stress. Additionally, when the

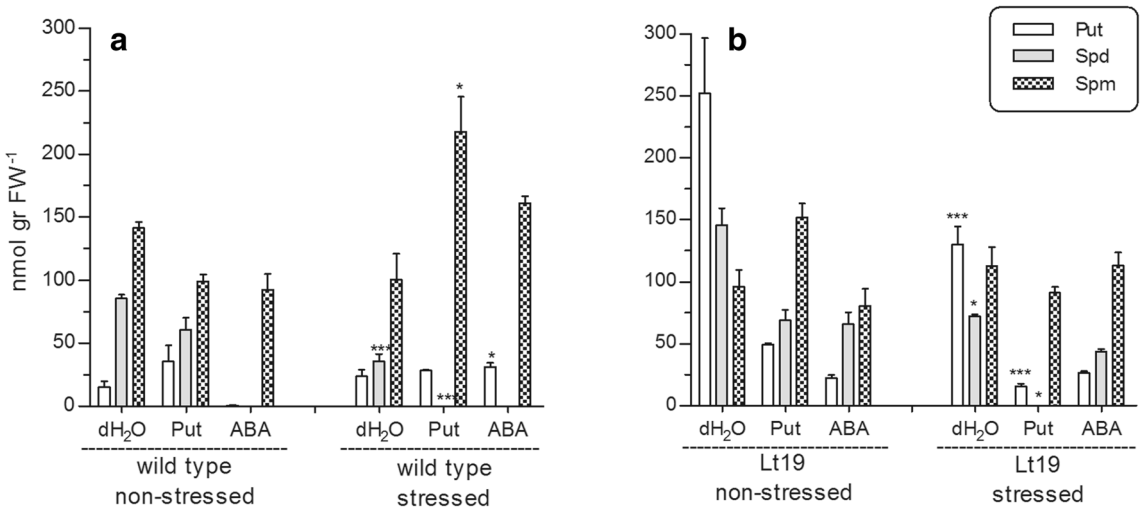

Fig. 4 Endogenous content of free putrescine, spermidine and spermine in the leaves of non-stressed and stressed plants sprayed with distilled water (dH_2O), Put or ABA. **a** and **b** wild-type and Lt19 plants, respectively. The *values* represent the mean ($n = 3$) ± SEM. *Asterisks* indicate significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) with respect to the non-stress control treatment

Table 1 Effect of NaCl on stomatal conductance, transpiration, water status, osmotic adjustment and membrane stability of shoots from wild-type and RD29A:oatADC (Lt19) transgenic plants grown for 35 days under normal and salt stress conditions

	Wild-type		RD29A:oatADC	
	Control	Salt stress	Control	Salt stress
Leaf RWC (%)	89.7 ± 1.2	86.97 ± 8.13	92.3 ± 3.08	80.53 ± 11.78
P_N (μmol CO_2 m^{-2} s^{-1})	2.70 ± 0.28	1.66 ± 0.33*	1.96 ± 0.27	0.55 ± 0.07**
g_s (mmol H_2O m^{-2} s^{-1})	0.04 ± 0.004	0.023 ± 0.005*	0.019 ± 0.004	0.004 ± 0.001**
E (mol H_2O m^{-2} s^{-1})	1.06 ± 0.10	0.57 ± 0.13*	0.44 ± 0.09	0.11 ± 0.02**
Leaf ψ_π^{100} (MPa)	−2.36 ± 0.21	−2.7 ± 0.05	−1.68 ± 0.14	−3.65 ± 0.31**
Leaf $\Delta\psi_\pi^{100}$		−0.34 ± 0.05		−1.97 ± 0.31**
Chlorophyll a	0.18 ± 0.02	0.15 ± 0.03	0.33 ± 0.1	0.23 ± 0.07
Chlorophyll b	0.29 ± 0.04	0.23 ± 0.05	0.51 ± 0.16	0.36 ± 0,11
Total chlorophylls	0.65 ± 0.09	0.55 ± 0.13	1.2 ± 0.61	0,83 ± 0,3
Leaf proline (μmol mg^{-1} FW)	2.53 ± 0.2	9.17 ± 0.83*	0.28 ± 0.08	6.71 ± 0.5***
Electrolyte leakage (%)	4.05 ± 0,45	2.35 ± 0,25	4.93 ± 0,03	1.6 ± 0.6*
MDA (nmol g^{-1} FW)	37.5 ± 1.5	27.6 ± 7.1	30.2 ± 0.8	40.9 ± 3.1*

Values are means ± SEM of three plants

Leaf RWC leaf relative water contents, *A* net photosynthesis rate, *gs* stomatal conductance, *E* transpiration rate, Ψ_π^{100} leaf osmotic potential, $\Delta\Psi_\pi^{100}$ leaf osmotic adjustment, *MDA* Malondialdehyde content

Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) among treatments within the day of measurement based on *t* test

Table 2 Sodium and potassium homeostasis on non-stressed and salt-stressed wild-type and transgenic (Lt19) plants weekly sprayed with distilled water (dH_2O), putrescine or ABA

			Control (dH_2O)		Putrescine		ABA	
			Control	Salt stress	Control	Salt stress	Control	Salt stress
wt	Roots	Na	8.1 ± 2.1	33.2 ± 6.0***	1.7 ± 0.1	16.8 ± 1.1**	1.8 ± 0.2	5.4 ± 0.4
		K	17.5 ± 4.3	21.7 ± 1.9	36.5 ± 0.7	22.2 ± 1.9	22.9 ± 0.2	18.2 ± 0.7
		Na/K	0.7 ± 0.3	1.5 ± 0.2***	0.04 ± 0.00	0.76 ± 0.06	0.08 ± 0.01	0.3 ± 0.01
	Stems	Na	4.0 ± 1.1	10.8 ± 1.0**	2.4 ± 0.01	22.9 ± 2.5***	3.7 ± 0.2	7.9 ± 0.02
		K	40 ± 5.7	34.1 ± 4.6	42.1 ± 2.4	32.7 ± 0.9	36.3 ± 1.4	51.2 ± 4.3
		Na/K	0.12 ± 0.0	0.35 ± 0.06	0.05 ± 0.00	0.7 ± 0.09*	0.1 ± 0.001	0.15 ± 0.01
	Leaves	Na	3.0 ± 0.5	14.6 ± 1.5***	3.1 ± 0.5	37.6 ± 4.5***	2.3 ± 1.1	7.6 ± 0.4
		K	38.1 ± 4.1	43.3 ± 4.2	22.3 ± 3.3	83.3 ± 5.5***	43.0 ± 0.9	47.1 ± 1.8
		Na/K	0.08 ± 0.0	0.33 ± 0.05**	0.14 ± 0.04	0.45 ± 0.02	0.05 ± 0.02	0.2 ± 0.002
Lt19	Roots	Na	9.5 ± 1.6	24.9 ± 2.6***	2 ± 0.02	24.6 ± 0.4***	2.5 ± 0.08	26.7 ± 0.9**
		K	19.3 ± 2.9	41.8 ± 5.5*	11.1 ± 0.84	32.8 ± 0.23**	20.8 ± 0.2	31.5 ± 4.7*
		Na/K	0.5 ± 0.08	0.63 ± 0.12	0.2 ± 0.01	0.76 ± 0.06**	0.12 ± 0.003	0.89 ± 0.16*
	Stems	Na	2.7 ± 0.3	18.4 ± 7.2*	2.2 ± 0.2	12.2 ± 1**	1.5 ± 0.3	12.9 ± 0.2**
		K	38.2 ± 4.2	37.7 ± 4.4	25.5 ± 0.8	41.3 ± 0.6***	35.5 ± 1.33	44.1 ± 0.6**
		Na/K	0.07 ± 0.004	0.6 ± 0.26*	0.08 ± 0.01	0.3 ± 0.02*	0.04 ± 0.01	0.3 ± 0.007**
	Leaves	Na	2.0 ± 0.4	19.7 ± 8.6*	1.9 ± 0.2	17 ± 0.27***	2.8 ± 0.01	19.7 ± 0.08***
		K	25.7 ± 3.0	29.0 ± 4.5	25.6 ± 0.3	23 ± 0.6	44.5 ± 2.2	26.6 ± 0.4*
		Na/K	0.07 ± 0.008	0.72 ± 0.31*	0.07 ± 0.01	0.74 ± 0.03**	0.06 ± 0.003	0.74 ± 0.001**

Na and K in mg g^{-1} DW

Values are means ± SEM of three plants

Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) among treatments within the day of measurement based on the *t* test

wt plants were weekly sprayed with Put, the amount of K^+ in the leaves increased 3.7-fold with respect to the non-stressed wt control plant. Likewise, the K^+ content increased after salt treatment and reached 41.8 ± 5.5 mg g^{-1} DW in the roots of the transgenic line. This concentration is 2.2-fold higher than the K^+ concentration of the non-stressed Lt19 control plants. The increased level of K^+ in the roots, which was concomitant with the lower accumulation of Na^+, enabled the Na^+/K^+ ratio to be in a similar range as that of the control treatment in the transgenic line. An exogenous supply of Put or ABA increased the accumulation of K^+ in the roots and stems of this genotype. Finally, the concentration of both monovalent cations remained unchanged in the whole stressed wt plants (roots, stems and leaves) treated with ABA.

Effect of Salt Stress on Sprouting and Dry Matter Production in Plants

The average number of new shoots formed per plant significantly decreased ($P < 0.01$) in the stressed wt plants (Table 3). Simultaneously, root growth, expressed as dry weight, dropped by 49.4 ± 7.7% ($P < 0.01$). Therefore, the shoot/root ratio increased substantially ($P < 0.05$) at the expense of roots. In contrast, the stressed transgenic plants showed a smaller reduction in shoot biomass and a slight promotion in root growth.

Discussion

We previously reported that the overexpression of oat *ADC* improved the water balance of *L. tenuis* plants subjected to drought by adjusting the leaf osmotic potential via the release of proline and by adapting the growth pattern of the entire plant through stimulating the development of roots at the expense of leaf and stem growth. Additionally, we determined that PA promote the expression of the ABA biosynthesis-related enzyme NCED under stress. Furthermore, the higher *NCED* gene expression promoted the enzymatic activity and increased the final product of the reaction, ABA (Espasandin and others 2014).

In the present study, we found that the overexpression of *ADC* correspondingly improved salt tolerance in plants subjected to a gradual increase in salinity (Fig. 5). In such an environment, the Lt19 plants were healthier than the wt plants, improving the shoot/root ratio in response to the adverse situation. In addition to the morphological advantages, the transgenic plants combined stomatal closure, osmotic adjustment and K^+ uptake to preserve cell turgor and protect the integrity of the cell membranes. The

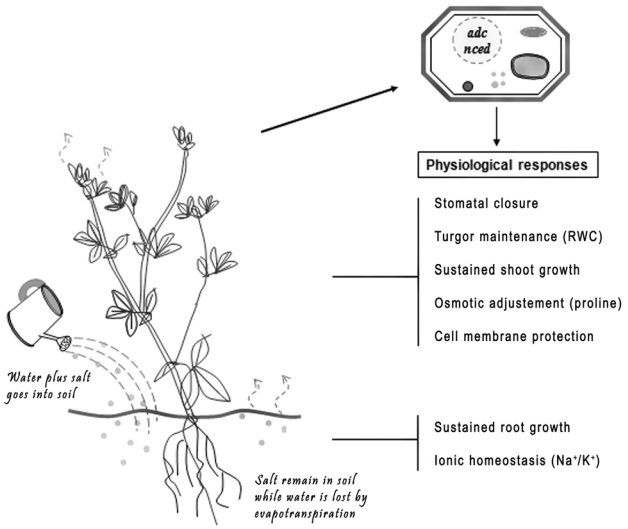

Fig. 5 A schematic diagram of the morphological, biochemical and physiological responses of the *pRD29A:oatADC* Lt19 line subject to salt stress

Table 3 Effect of salinity on shoot branching, shoot dry weight and root dry weight of wild-type and RD29A::oatADC (Lt19) transgenic plants grown for 35 days under normal and stress conditions

	Wild-type		RD29A::oatADC	
	Control	Salt stress	Control	Salt stress
Shoot branching[1]	18.3 ± 1.2[2]	8.3 ± 3.7**	29.7 ± 4.0	17.2 ± 5.9
Shoot dry weight[3] (g)	2.1 ± 0.3	1.1 ± 0.3	3.1 ± 0.7	2.2 ± 0.2
Root dry weight (g)	0.7 ± 0.0	0.2 ± 0.0**	0.7 ± 0.1	0.8 ± 0.2
Shoot/root ratio	2.9 ± 0.3	7.2 ± 2.7*	4.1 ± 0.8	3.4 ± 1.4
Shoot branching variation (%)		−54.5 ± 20.5		−48.4 ± 20.4
Shoot weight variation (%)		−49.4 ± 7.7		−27.9 ± 7.9
Root weight variation (%)		−78.1 ± 1.6		7.4 ± 3.3

[1]Shoots branching: mean number of new shoots formed per plant

[2]Values are means ± SEM of three plants

[3]Including leaves and stems

Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) among treatments within the day of measurement based on the *t* test

salinity treatment increased the K^+ content of the roots of Lt19 stressed plants, concomitantly with a decreased accumulation of Na^+, balancing the Na^+/K^+ ratio. Similarly, our results revealed an increase in the expression and enzymatic activity of oat ADC in response to stress; the free Put level decreased when the electrical conductivity of the soil increased to 30.3 ± 11.1 dS m^{-1}.

Although the intrinsic contributions of PA to stress protection remain to be analysed, our results and those from previous studies suggest that the accumulation of free Put occurs early in the stress response to dehydration (Maiale and others 2004), and diminished when the concentrations of cations in the cytoplasm increase (Santa Cruz and others 1997; Zarza and others 2016). The elevated levels of endogenous PA can be rapidly converted into each other in the PA cycle (Pál and others 2015). Several studies have suggested that soluble and insoluble PA and the products of PA oxidation are required for different processes in plant development and stress responses (Janicka Russak and others 2010; Zhang and others 2014). Our results demonstrated that the amount of conjugated and insoluble bound Put was higher in the Lt19 line than in wt plants subjected to salt stress, suggesting that the putative function of Put should be related to its conjugation with channels because PA have a strong positive charge and can effectively block cation channels (Zepeda-Jazo and others 2011). On the other hand, no damage at the level of cell membranes was detected, the increase in MDA content can be explained by the reduction in the level of free Put due to DAO activity yielding pyrroline, H_2O_2 and ammonia (Moschou and others 2012; Song and others 2015). Likewise, PA have been associated with other metabolic pathways, such as the Krebs and nitrogen cycles (Moschou and others 2012; Zarza and others 2016). Further metabolic profiling analyses of the hormone and stress-signaling mutants should provide information on the regulatory circuits that modulate PA metabolism and their coordinated action with other metabolic pathways (Tiburcio and others 2014).

We detected a strong osmotic adjustment in the leaves of the Lt19 line consistent with a significant increase in the leaf proline content. The increase in the proline levels generally occurs later than the changes in the levels of PA, suggesting a regulatory mechanism in PA metabolism for proline biosynthesis (Pál and others 2015; Tonon and others 2004), which contributes to the stabilization of the cell membrane and helps to preserve enzymatic activities under salt stress (Sharma and others 2011). The use of an osmoregulation mechanism, which is coupled with a reduction in the stomatal conductance, to counteract the deleterious effects of osmotic stress and the redistribution of photoassimilates supporting root growth suggests that the transgenic line is more stress tolerant than the wt plants (Wang and others 2011). Along these lines, Liu and others (2000) reported that Put may be positively involved with stomatal regulation in wheat and may help the plant to reduce water loss under stress conditions by enhancing stomatal closure as a result of the direct interaction of Put with voltage-dependent K^+ inward rectifier channels in guard cells.

Due to the chemical similarity of Na^+ and K^+, K^+ homeostasis is severely influenced by salt stress. High Na^+ concentrations in plants often cause K^+-deficiency symptoms and interrupt many physiological processes mediated by K^+ ions, including enzymatic reactions and turgor function (Yao and others 2010). The maintenance of an adequate Na^+/K^+ ratio is considered to be vital for plant survival. In this sense, an improvement in ion homeostasis was observed in the roots of Lt19 plants subjected to salt stress via a decrease in the Na^+ level in combination with a slight increase in K^+ concentration, which enabled the maintenance of the Na^+/K^+ ratio. Na^+ mainly accumulated in the leaves, a result that is attributed to a better compartmentalization in shoots as a mechanism of tissue tolerance (Munns and Tester 2008). Furthermore, compatible solutes and bound PA have also been suggested to improve the Na^+/K^+ ratio via the inhibition of non-selective cation channels and a consequent decline in membrane depolarization and K^+ efflux (Pál and others 2015; Shabala and Cuin 2007). Their specificity for selectively blocking outward Na^+ channels in tonoplasts helps the vacuole hold Na^+, thus changing the effective Na^+/K^+ ratio in the cytoplasm under stress (Zepeda-Jazo and others 2008; Janicka Russak and others 2010). Moreover, exogenous Put is associated with an increase in root growth, which has a positive effect on the cation discrimination in the process of absorption (decreasing Na^+/K^+ and Na^+/Ca^{2+} ratios) in roots (Shi and others 2008) or in the regulation of the translocation of ions in plants (Ndayiragije and Lutts 2006; Sharma and others 2011).

Finally, our results showed a significant decrease in the Na^+ concentration at the whole plant level in wt plants by increasing the Put accumulation, which was stimulated by the exogenous supply of ABA. A potential role of ABA in the induction of genes that encode PA biosynthetic enzymes under a wide range of stress and development conditions has previously been demonstrated (Alcázar and others 2010; Minocha and others 2014). The increase in the expression of the *NCED* gene from Lt19 plants subjected to salt stress concomitantly with the augmentation of Put content in the leaves of the stressed wt genotype following ABA treatment suggested that Put and ABA are integrated in a positive feedback loop in response to abiotic stress. However, whether the induction of Put/ABA biosynthetic genes or the accumulation of Put is associated directly with the ABA signaling cascade has not yet been addressed (Liu and others 2015). Notwithstanding, an *Arabidopsis* single

mutant of *ADC* with significantly reduced Put content and reduced expression of *NCED3* showed increased sensitivity to low temperature and had improved low temperature tolerance in complementation with ABA and Put (Cuevas and others 2008). Further metabolic profiling analyses of the hormone and stress-signaling mutants should provide information on the regulatory circuits that modulate PA metabolism and their coordinated action with other metabolic pathways (Tiburcio and others 2014).

In conclusion, our results provide new insights into the participation of PA in the biochemical and physiological processes of the response of *L. tenuis* that mitigates the deleterious effects of salt stress as well as the evidence to indicate that the overexpression of the *ADC* gene links the interaction of ABA with signal response and modulation.

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References

Alcázar R, Planas J, Saxena T, Zarza X, Bortolotti C, Cuevas J, Bitrián M, Tiburcio AF, Altabella T (2010) Putrescine accumulation confers drought tolerance in transgenic *Arabidopsis* plants overexpressing the homologous ADC2 gene. Plant Physiol Biochem 48:547–552. doi:10.1016/j.plaphy.2010.02.002

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72:248–254. doi:10.1016/0003-2697(76)90527-3

Calzadilla PI, Gazquez A, Maiale SJ, Ruiz OA, Menéndez AB (2014) Polyamines as indicators and modulators of the abiotic stress in plants. In: Anjum NA, Singh SS, Gill R (eds) Plant adaptation to environmental change: significance of amino acids and their derivatives. CAB International, Wallingford, pp 109–128. doi:10.1079/9781780642734.0109

Calzadilla PI, Maiale SJ, Ruiz OA, Escaray FJ (2016) Transcriptome response mediated by cold stress in *Lotus japonicus*. Front Plant Sci 7:374. doi:10.3389/fpls.2016.00374

Cuevas JC, Lopez-Cobollo R, Alcázar R, Zarza X, Koncz C, Altabella T, Salinas J, Tiburcio AF, Ferrando A (2008) Putrescine is involved in *Arabidopsis* freezing tolerance and cold acclimation by regulating abscisic acid levels in response to low temperature. Plant Physiol 148:1094–1105. doi:10.1104/pp.108.122945

Espasandin FD, Collavino MM, Luna CV, Paz RC, Tarragó JR, Ruiz OA, Mroginski LA, Sansberro PA (2010) *Agrobacterium tumefaciens*-mediated transformation of *Lotus tenuis* and regeneration of transgenic lines. Plant Cell Tiss Organ Cult 102:181–189. doi:10.1007/s11240-010-9720-x

Espasandin FD, Maiale S, Calzadilla P, Ruiz OA, Sansberro PA (2014) Transcriptional regulation of 9-*cis*-epoxycarotenoid dioxygenase (NCED) gene by putrescine accumulation positively modulates ABA synthesis and drought tolerance in *Lotus tenuis* plants. Plant Physiol Biochem 76:29–35. doi:10.1016/j.plaphy.2013.12.018

Flowers TJ (2004) Improving crop salt tolerance. J Exp Bot 55:307–319. doi:10.1093/jxb/erh003

Garriz A, Dalmasso MC, Marina M, Rivas EI, Ruiz OA, Pieckenstain FL (2004) Polyamine metabolism during the germination of *Sclerotinia sclerotiorum* ascospores and its relation with host infection. New Phytol 161:847–854. doi:10.1046/j.1469-8137.2003.00983.x

Gill SS, Tuteja N (2010) Polyamines and abiotic stress tolerance in plants. Plant Signal Behav 5:26–33. doi:10.1016/j.jplph.2009.04.016

Hodges DM, De Long JM, Forney CF, Prange RK (1999) Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta 207:604–611. doi:10.1007/s004250050524

Hussain SS, Ali M, Ahmad M, Siddique KHM (2011) Polyamines: natural and engineered abiotic and biotic stress tolerance in plants. Biotechnol Adv 29:300–311. doi:10.1016/j.biotechadv.2011.01.003

Janicka Russak M, Kabala K, Mlodzinska E, Klobus G (2010) The role of polyamines in the regulation of the plasma membrane and the tonoplast proton pumps under salt stress. J Plant Physiol 167:261–269. doi:10.1016/j.jplph.2008.01.003

Liu K, Fu H, Bei Q, Luan S (2000) Inward potassium channel in guard cells as a target for polyamine regulation of stomatal movements. Plant Physiol 124:1315–1326. doi:10.1104/pp.124.3.1315

Liu J-H, Wang W, Wu H, Gong X, Moriguchi T (2015) Polyamines function in stress tolerance: from synthesis to regulation. Front Plant Sci 6:827. doi:10.3389/fpls.2015.00827

Magné C, Larher F (1992) High sugar content of extracts interferes with colorimetric determination of amino acids and free proline. Anal Biochem 200:115–118. doi:10.1016/0003-2697(92)90285-F

Maiale S, Sánchez DH, Guirado A, Vidal A, Ruiz AO (2004) Spermine accumulation under salt stress. J Plant Physiol 161:35–42. doi:10.1078/0176-1617-01167

Marco F, Alcázar R, Tiburcio AF, Carrasco P (2011) Interactions between polyamines and abiotic stress pathway responses unravelled by transcriptome analysis of polyamine overproducers. Omics 15:775–781. doi:10.1089/omi.2011.0084

Minocha R, Majumdar R, Minocha SC (2014) Polyamines and abiotic stress in plants: a complex relationship. Front Plant Sci 5:175. doi:10.3389/fpls.2014.00175

Moschou PN, Wu J, Cona A, Tavladoraki P, Angelini R, Roubelakis-Angelakis KA (2012) The polyamines and their catabolic products are significant players in the turnover of nitrogenous molecules in plants. J Exp Bot 63:5003–5015. doi:10.1093/jxb/ers202

Munns R, Tester M (2008) Mechanisms of salinity tolerance. Plant Biol 59:651–681. doi:10.1146/annurev.arplant.59.032607.092911

Ndayiragije A, Lutts S (2006) Do exogenous polyamines have an impact on the response of a salt-sensitive rice cultivar to NaCl? J Plant Physiol 163:506–516. doi:10.1016/j.jplph.2005.04.034

Pál M, Szalai G, Janda T (2015) Speculation: polyamines are important in abiotic stress signaling. Plant Sci 237:16–23. doi:10.1016/j.plantsci.2015.05.003

Rhoades JD, Manteghi NA, Shouse PJ, Alves WJ (1989) Soil electrical conductivity and soil salinity: new formulations and calibrations. Soil Sci Soc Am J 53:433–439. doi:10.2136/sssaj1989.03615995005300020020x

Rodríguez-Kessler M, Ruiz OA, Maiale S, Ruiz-Herrera J, Jiménez-Bremont JF (2008) Polyamine metabolism in maize tumours induced by *Ustilago maydis*. Plant Physiol Biochem 46:805–814. doi:10.1016/j.plaphy.2008.05.012

Roy SJ, Negrao S, Tester M (2014) Salt resistant crop plants. Curr Opin Biotechnol 26:115–124. doi:10.1016/j.copbio.2013.12.004

Santa Cruz A, Acosta M, Pérez-Alfocea F, Bolarin MC (1997) Changes in free polyamine levels induced by salt stress in leaves of cultivated and wild tomato species. Physiol Plant 101:341–346. doi:10.1111/j.1399-3054.1997.tb01006.x

Shabala S, Cuin TA (2007) Potassium transport and plant salt tolerance. Physiol Plant 133:651–669. doi:10.1111/j.1399-3054.2007.01008.x

Sharma DK, Dubey AK, Srivastav M, Singh AK, Sairam RK, Pandey RN, Dahuja A, Kaur C (2011) Effect of putrescine and paclobutrazol on growth, physiochemical parameters, and nutrient acquisition of salt-sensitive citrus rootstock Karna khatta (*Citrus karna* Raf.) under NaCl stress. J Plant Growth Regul 30:301–311. doi:10.1007/s00344-011-9192-1

Shavrukov Y (2013) Salt stress or salt shock: which genes are we studying? J Exp Bot 64:119–127. doi:10.1093/jxb/ers316

Shi K, Huang YY, Xia XJ, Zhang YL, Zhou YH, Yu JQ (2008) Protective role of putrescine against salt stress is partially related to the improvement of water relation and nutritional imbalance in cucumber. J Plant Nutr 31:1820–1831. doi:10.1080/01904160802325446

Song Y, Diao Q, Qi H (2015) Polyamine metabolism and biosynthetic genes expression in tomato (*Lycopersicon esculentum* Mill.) seedlings during cold acclimation. Plant Growth Regul 75:21–32. doi:10.1007/s10725-014-9928-6

Tang W, Newton RJ (2005) Polyamines reduce salt-induced oxidative damage by increasing the activities of antioxidant enzymes and decreasing lipid peroxidation in *Virginia pine*. Plant Growth Regul 46:31–43. doi:10.1007/s10725-005-6395-0

Tiburcio AF, Altabella T, Bitrián M, Alcázar R (2014) The roles of polyamines during the lifespan of plants: from development to stress. Planta 240:1–18. doi:10.1007/s00425-014-2055-9

Tonon G, Kevers C, Faivre-Rampant O, Graziani M, Gaspar T (2004) Effect of NaCl and mannitol iso-osmotic stresses on proline and free polyamine levels in embryogenic *Fraxinus angustifolia* callus. J Plant Physiol 161:701–708. doi:10.1078/0176-1617-01096

Vernon LP (1960) Spectrophotometric determination of chlorophylls and pheophytins in plant extracts. Anal Chem 32:1144–1150. doi:10.1021/ac60167a041

Verslues PE, Agarwal M, Katiyar-Agarwal S, Zhu J, Zhu JK (2006) Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. Plant J 45:523–539. doi:10.1111/j.1365-313X.2005.02593.x

Wang J, Sun P, Chen C, Wang Y, Fu X, Liu JH (2011) An arginine decarboxylase gene PtADC from *Poncirus trifoliata* confers abiotic stress tolerance and promotes primary root growth in *Arabidopsis*. J Exp Bot 62:2899–2914. doi:10.1093/jxb/erq463

Yao X, Horie T, Xue S, Leung H-Y, Katsuhara M, Brodsky D, Wu Y, Schroeder J (2010) Differential sodium and potassium transport selectivities of the rice OsHKT2;1 and OsHKT2;2 transporters in plant cells. Plant Physiol 152:341–355. doi:10.1104/pp.109.145722

Zarza X, Atanasov KE, Marco F, Arbona V, Carrasco P, Kopka J, Fotopoulos V, Munnik T, Gómez-Cadenas A, Tiburcio AF, Alcázar R (2016) Polyamine oxidase 5 loss-of-function mutations in *Arabidopsis thaliana* trigger metabolic and transcriptional reprogramming and promote salt stress tolerance. Plant Cell Environ. doi:10.1111/pce.12714

Zepeda-Jazo I, Shabala S, Chen Z, Pottosin II (2008) Na–K transport in roots under salt stress. Plant Signal Behav 3:401–403. doi:10.4161/psb.3.6.5429

Zepeda-Jazo I, Velarde-Buendia AM, Enrıquez-Figueroa R, Bose J, Shabala S, Muñiz-Murguia J, Pottosin I (2011) Polyamines interact with hydroxyl radicals in activating Ca^{2+} and K^{+} transport across the root epidermal plasma membranes. Plant Physiol 157:2167–2180. doi:10.1104/pp.111.179671

Zhang G, Xu S, Hu Q, Mao W, Gong Y (2014) Putrescine plays a positive role in salt-tolerance mechanisms by reducing oxidative damage in roots of vegetable soybean. J Integr Agric 13:349–357. doi:10.1016/S2095-3119(13)60405-0

Zhou F, Sosa J, Feldmann K (2007) High throughput approaches for the identification of salt tolerance genes in plants. In: Jenks MA, Hasegawa PM, Jain SM (eds) Advances in molecular breeding toward drought and salt tolerant crops. Springer, The Netherlands, pp 359–379. doi:10.1007/978-1-4020-5578-2_15