Research article

Transcriptional regulation of 9-*cis*-epoxycarotenoid dioxygenase (NCED) gene by putrescine accumulation positively modulates ABA synthesis and drought tolerance in *Lotus tenuis* plants

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ABSTRACT

The accumulation of putrescine (Put) and increased arginine decarboxylase (ADC, EC 4.1.1.19) activity levels in response to osmotic stress has been reported; however, the biological meaning of this increase remains unclear. To obtain new insights into these questions, we studied the drought response of a transgenic *Lotus tenuis* line that expresses the oat ADC gene, which is driven by the stress-inducible *pRD29A* promoter. This line contains high levels of Put with no changes in spermidine and spermine contents, even under water deficits. Our results indicate that the biochemical and morphological responses to dehydration correlate with the Put level and provide evidence that Put controls the ABA content in response to drought by modulating ABA biosynthesis at the transcriptional level.

1. Introduction

Polyamines (PAs) are small protonated compounds with key roles in plant development and stress protection (Urano et al., 2003; Hussain et al., 2011), of which the most predominant forms are the diamine putrescine (Put), triamine spermidine (Spd) and tetramine spermine (Spm) (Marco et al., 2011). In higher plants, there are two main pathways for the biosynthesis of PAs (Bae et al., 2008). In eukaryotic cells, Put is synthesised directly from ornithine through the activity of ornithine decarboxylase (ODC). In plants, there is an alternative pathway for Put synthesis via the activity of arginine decarboxylase (ADC; EC 4.1.1.19). Higher molecular weight PAs (Spd and Spm) are formed by the sequential addition of

aminopropyl groups to Put (Marco et al., 2011). Frequently, the genes that are involved in the biosynthesis of PAs are regulated in response to adverse environmental conditions. In fact, both ODC and ADC are known to be induced by chilling (Hao et al., 2005), osmotic stress (Hao et al., 2005; Bouchereau et al., 1999; Soyka and Heyer, 1999), acidic pH (Nam et al., 1997), and nutrient deficiency (Malmberg et al., 1998). Usually, as a result of an increase in the enzyme activity, Put content increases at the expense of Spm and Spd, which enhances desiccation tolerance (Capell et al., 2004; Zapata et al., 2008; Alet et al., 2011). Additionally, the increased content of PAs by the overexpression of genes that encode ADC, spermidine synthase (SPDS), or S-adenosylmethionine decarboxylase (SAMDC), as well as resulting from exogenous applications, reveals that PAs strong promote the expression of functional and regulatory genes that are involved in stress tolerance (Gill and Tuteja, 2010). In this context, PAs maintain cell stability and ion homeostasis (Legocka and Kluk, 2005), increase the activity of antioxidant enzymes that protect the photosynthetic apparatus (Gill and Tuteja, 2010), control the movement of stomata, and regulate root growth (Wang et al., 2011). Finally, PAs are involved in signal transduction by interacting with other hormones (Bitrián et al., 2012), such as ABA (Hussain et al., 2011; Alet et al., 2011; Alcázar et al., 2006a; Kuznetsov et al., 2006; Liu et al., 2007),

ethylene (Kaur-Sawhney et al., 2003), methyl jasmonate (Peremarti et al., 2010), cytokinins (Rakova and Romanov, 2005) and gibberellins (Alcázar et al., 2005).

The overexpression of genes that are associated with the biosynthesis of PAs by the use of a stress-inducible promoter has become a valuable tool to study their roles in the mechanisms that are involved in plant responses to stress conditions (Alet et al., 2011). To understand how Put levels are involved in the response of *Lotus tenuis* to water deficits, we previously obtained transgenic plants that overexpress the oat ADC gene under the control of the *Arabidopsis thaliana* stress-inducible promoter *pRD29A* (Espasandin et al., 2010). The *pRD29A* promoter presents DRE (Drought Responsive Element) and ABRE (ABA Responding Element) cis-acting elements, which enable high and efficient induction under ABA and osmotic stress. In this study, we demonstrate that the endogenous Put level affects the biochemical and physiological response of *L. tenuis* to drought and how this polyamine potentially modulates ABA-dependent responses.

2. Materials and methods

2.1. Plant materials

Assays were performed with *L. tenuis* cv INTA PAMPA. Micropropagated plants of similar ages and sizes from wild type and transgenic lines that harboured the *pRD29A*::oat ADC construct, which were obtained by transformation via *Agrobacterium tumefaciens* (Espasandin et al., 2010), were placed in 2 l pots that were filled with a mixture of sterile soil and perlite (1/1 w/w), as well as 0.5 g of controlled release micro-fertiliser (Osmocote; N/P/K, 9/45/15; 180-day release), and were grown for 6–8 weeks under a day/night air temperature of 25–27/20–22 °C, respectively, and a substrate temperature of 22–25 °C. A 14 h light/10 h dark photoperiod was maintained, and light was provided by 16 cool-white fluorescent lamps (40 W; distance above plants 1.6 m) at an intensity of 345 μmol m^{-2} s^{-1} PPFD in the wavelength range of 400–700 nm.

2.2. Drought assay

Plants were irrigated to the drip point ($\Psi_{soil} = -0.04$ MPa) at the beginning of the experiment and subsequently subjected to a continuous soil drying episode by withholding water from pots until the Ψ_{soil} at pre-dawn reached −2 MPa. Pots were covered with aluminium foil to prevent evaporation. A well-watered (control) was included. Ψ_{soil} was measured with a PST-55 thermocouple (Wescor Inc., Utah, USA) that was placed horizontally at the root zone 8 cm below the soil surface, close to the centre of the pot. When the $\Psi_{soil} = -2$ MPa, shoot tissues (stems and leaves) were harvested per plant to obtain three samples for osmotic potential (Ψ_{π}^{100}) measurement and three samples for RWC measurement. The mean value from the two subsamples per plant was used to characterise each plant. For Ψ_{π}^{100} measurement, the leaves were rehydrated to constant fresh weight by placing the leaves in a beaker of distilled water under controlled environmental conditions, then placing the leaves in a syringe, freezing in liquid N$_2$ and storing at −80 °C pending further analysis. Syringes were thawed until samples reached room temperature, and the Ψ_{π}^{100} of the expressed sap was measured with a C-52 thermocouple. Leaf RWC was determined using the following formula: leaf RWC (%) = (FW-DW)/(SW-DW) × 100. Leaf osmotic adjustment ($\Delta\psi_{\pi}^{100}$) during the drying period was computed as the difference between pre-drought and drought Ψ_{π}^{100}. Leaf stomatal conductance (g) and transpiration (E) were measured in intact, fully expanded mature leaves with a LI-6400 portable photosynthesis meter.

Measurements were taken at mid-day using healthy, fully expanded leaves at the same developmental stage. Sample data were calculated from three biological replicates.

2.3. Exogenous treatments

To validate the response of transgenic lines, wild type plants were sprayed with either distilled water (control), putrescine dihydrochloride 100 ppm (98% purity), (±) cis, trans-ABA 100 ppm (99% purity) or tungstate sodium dihydrate (99% purity) 100 ppm, until incipient runoff (approximately 5 ml of aqueous solution per plant). All chemicals that were used were obtained from Sigma Aldrich (St. Louis, MO, USA). The solutions included Triton (0.1%) as a surfactant and a minimum amount of ethanol, which was used to dissolve the ABA. Sprays were repeated once a week until Ψ_{soil} reached −2 MPa.

2.4. Enzyme activity assays

The ADC (EC 4.1.1.19) was extracted by the homogenisation of shoot (stem and leaves) tissues (500 mg fresh weight) in 2 volumes of 100 mM phosphate buffer (pH 7.5), which contained 0.5 mM EDTA, 10 mM dithiothreitol, 1 mM pyridoxal phosphate and 20 mM sodium ascorbate. The crude extracts that were thus obtained were clarified by centrifugation at 10,000 g for 10 min. All of the above-described procedures were performed at 4 °C. The protein concentration in the supernatants was determined using the standard method of Bradford (Bradford, 1976). Enzyme activities were determined by mixing 190 ml of the extract with 10 ml of the substrate solution in a glass tube that was fitted with a rubber stopper and a filter paper disc that was soaked in 2 N KOH. Substrate solutions for the determination of ADC activities contained 1 mM nonradioactive substrate, which was amended with 5 nCi ml-1 L-[$^{14}C_1$]arginine. After 1-h incubation at 37 °C, the reaction was stopped, and $^{14}CO_2$ was released by adding 200 ml of 10% (v/v) perchloric acid. Following a 1-h distillation of $^{14}CO_2$ at 37 °C, the paper was immersed in 200 ml scintillation cocktail (4 g Omnifluor in toluene), and radioactivity was determined using a Beckman LS 5000 scintillation counter (Beckman Coulter, Brea, CA).

2.5. Free polyamine quantification

To determine free Put, Spm and Spd levels in leaf extracts, plant material (300 mg) was ground in liquid nitrogen, extracted in 600 μl 5% (v/v) perchloric acid and incubated overnight at 4 °C. After centrifugation at 10,000 g for 15 min, 10 μl of 100 mM 1.7 heptanediamine (ICN Biomedicals, Costa Mesa, CA) was added as an internal standard to 200 μl aliquots of leaf extracts, followed by the addition of 200 μl saturated Na$_2$CO$_3$ and 400 μl dansyl chloride (10 mg ml^{-1} in acetone); the mixture was then incubated overnight in the dark at room temperature. The reaction was stopped by adding 100 μl of Proline (100 mg ml^{-1}), and dansylated amine was extracted in 500 μl of toluene. The organic phase was vacuum-evaporated, and dansylated polyamines were dissolved in 200 μl of acetonitrile and analysed by reversed phase high-performance liquid chromatography as described previously by Garriz et al. (2004). Proline was determined spectrophotometrically by the ninhydrin reaction (Magné and Larher, 1992).

2.6. Free ABA, ABA-glucosyl ester, and phaseic acid quantification

The determination of free ABA, ABA-glucosyl ester (ABA-GE), and phaseic acid was performed by liquid chromatography tandem mass spectrometry (LC-MS/MS), following the methodology that was proposed by Chiwocha et al. (2003), with minor modifications.

Plant material was collected at mid-day, frozen in liquid nitrogen and dehydrated. Leaf tissue (200 mg DW) was extracted for 16 h at 4 °C with H_2O:HOAc (99:1 at pH: 2–3). Subsequently, 50 ng of $^2H_6$-ABA, $^2H_5$-ABA-GE, and $^2H_3$-PA were added as internal standard. After 30 min (to allow isotope equilibration), the sample was filtered, and the methanol was evaporated under low pressure. The remaining aqueous fraction was filtered and centrifuged for 10 min at 5000 rpm. The supernatant was adjusted to pH 3, partitioned two times with ethyl acetate that was saturated with aqueous acetic acid (0.5%) and evaporated to dryness. Afterwards, the sample was resuspended in methanol for purification with a C18 cartridge (Sep-Pak, Waters-Millipore) and brought to dryness using a SpeedVac. Finally, each sample was dissolved in 50 L of MeOH (100%) for identification and quantification by LC-MS/MS. Ten microlitres was injected into a liquid chromatograph (Alliance 2695, Waters Inc.), which was equipped with a Restek C18 column (2.1 × 100 mm, 5 microns), at 25 °C and eluted with a methanol/water (0.2% acetic acid) gradient (40% MeOH: 60–0.2H_2O:HOAc) at a flow rate of 0.2 ml min^{-1} through a double quadrupole mass spectrometer (Quatro Ultimatm^{tm} Pt., Micromass, UK), which used the ion source in the negative mode and capillary electrospray ionisation (ESI). Compounds were identified by the comparison of retention times with the internal standard, and the ions were monitored by multiple reaction (MRM function) according to the molecular masses and the mass transitions for ABA and $^2H_6$-ABA$_1$ 263 > 153 and 269 > 159, whereas for ABA-GE and $^2H_5$-ABA/GE: 425 > 263 and 430 > 268, for PA and $^2H_3$-PA 279 > 139 and 282 > 142, respectively. Quantification was performed by determining the area ratio between the endogenous and deuterated forms and by extrapolating on a calibration curve that was prepared with known concentrations.

2.7. Quantitative real-time PCR expression analysis

Total RNA was extracted with an SV Total RNA Isolation System (Promega) according to the manufacturer's instructions. In total, 1 µg of RNA was reverse transcribed to cDNA with random hexamers using SuperScriptTM III RT (Invitrogen) according to manufacturer's instructions.

Real-time qRT-PCR reactions were prepared using SYBR Green PCR Master Mix (Applied Biosystems, California, USA) according to the manufacturer's instructions. The reaction mix for each well included 2.5 µl cDNA, 10 µl nix of specific primers (FW and REV) and 12.5 µl 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 AtNCED3 5′- ATA GGG AAC CCT GGA TGG AA -3′ and 5′- GAG AAG GAA TGG AAA TCT GAG C -3′. *Poa* β −tubulin was amplified as a reference gene to normalise the expression levels. The β-tubulin primer sequences were 5′- GTG GAG TGG ATC CCC AAC AA -3′ and 5′- AAA GCC TTC CTC CTG AAC ATG G -3′. Three biological replicates were amplified in triplicate. The thermocycler program included the following steps: an initial denaturation (95 °C/10 min), which was followed by 40 cycles of 95 °C/30 s and 60 °C/1 min, using a 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 levels and the significance of the measurements.

2.8. Statistical analysis

The data were subjected to an ANOVA (GraphPad Software, San Diego, CA, USA) following Tukey's multiple comparison test to compare differences among treatments. To assess statistical significance, a probability level of 0.05 was chosen.

3. Results

3.1. Putrescine accumulation enhances drought tolerance

Transgenic *L. tenuis* plants that harbour a single insertion of *pRD29A*::oat ADC that has been integrated in their genome were previously obtained via *A. tumefaciens* transformation (Espasandin et al., 2010). The *Lt*19 line was selected for further study based on its higher ADC activity under water deficits. An osmotic stress signal was detected in leaves and stems of GUS transgenic lines that were driven by the *pRD29A* promoter. The *Lt*19 line exhibited the same phenotype as the untransformed controls under non-stress conditions.

Wild type (WT) and transgenic plants were subjected to a controlled experiment in which drought was applied gradually by restricting evaporation. This approach helped to circumvent artificial changes in gene expression and physiological responses, which may be induced by a shock treatment. The decrease in the soil water content significantly stimulated oat ADC gene expression in the *Lt*19 line (Table 1). Consequently, the enzymatic activity increased 2-fold, which tripled the level of free Put in shoots. No changes were observed in ADC activity or free Put content in wild type plants. Likewise, the levels of Spd and Spm in their active forms were unchanged in response to stress in both genotypes.

To understand the physiological process that underlies drought tolerance, we determined the water status and exchange of the *pRD29A*::ADC line (Table 2). The leaf relative water content values differ between the two genotypes under stress conditions. A minor decrease (close to 12%) was observed in transformed plants that were subjected to $\psi_{soil} = -2$ MPa; under the same conditions, the RWC of WT plants decreased by 18–20%. The stomatal conductance declined significantly in response to stress in both genotypes, and consequently, the rate of transpiration was reduced. Although the relative water content of shoots decreased in response to the water deficit and correlated with a sharp decline in stomatal conductance and transpiration, the RWC in *Lt*19 did not change significantly, decreasing by approximately 12% when the soil water potential dropped to −2 MPa. Concurrently, the osmotic potential in leaves of the transgenic line decreased from −1.68 to −2.74 MPa and displayed an osmotic adjustment that was close to −1 MPa. Under optimal soil moisture conditions, the transformed plants accumulated nine times less proline in their leaves than did the WT plants. In both genotypes, the water deficit caused significant increases in

Table 1
ADC mRNA expression, ADC activity, and shoot polyamines content of the osmotic-elicited *pBiRD29A*::oat ADC Lt19 line and wild-type grown in pots.

	Wild type		Lt19	
	Non-stressed	Stressed	Non-stressed	Stressed
OatADC Relative Expression qRT-PCR	1 ± 0.8	0.9 ± 0.1	1 ± 0.4	102.3 ± 20.9***
ADC activity nmol $^{14}CO_2$ h^{-1} mg protein	0.09 ± 0.01	0.08 ± 0.03	1.3 ± 0.2	2.9 ± 0.00**
Putrescine nmol gr FW^{-1}	15.3 ± 4.7	20.1 ± 3.8	40.9 ± 6.2	148.8 ± 3.7***
Spermidine nmol gr FW^{-1}	86 ± 3.0	77 ± 10.5	116 ± 28.9	64 ± 35.0
Spermine nmol gr FW^{-1}	141 ± 6.4	165 ± 53.7	180 ± 22.1	114 ± 56.2

Values = mean ± SEM; $n = 3$; simple, double and triple asterisks indicate significant differences respect to the non-stressed control treatment at $P < 0.05$, 0.001 and 0.0001, respectively; Tukey's multiple comparison test.

Table 2
Leaf relative water content, stomatal conductance, osmotic adjustment and transpiration in *pRD29A*::oatADC line and wild-type plants during dehydration.

	Wild type		Lt19	
	Non-stressed	Stressed	Non-stressed	Stressed
RWC (%)	89.7 ± 1.2	$70.2 \pm 4.5^*$	92.3 ± 3.08	80.2 ± 6.5
g (mmol H_2O m^{-2} s^{-1})	0.047 ± 0.000	$0.006 \pm 0.002^{***}$	0.068 ± 0.002	$0.009 \pm 0.001^{***}$
E (mol H_2O m^{-2} s^{-1})	1.15 ± 0.086	$0.16 \pm 0.03^{***}$	1.59 ± 0.11	$0.27 \pm 0.03^{***}$
ψ_π^{100} (MPa)	-2.36 ± 0.21	-2.7 ± 0.05	-1.68 ± 0.14	$-2.74 \pm 0.31^{**}$
$\Delta\psi_\pi^{100}$	–	-0.18 ± 0.12	–	$-1.06 \pm 0.31^{**}$
Leaf proline (μmol mg^{-1} FW)	2.53 ± 0.2	$13.2 \pm 2.14^{**}$	0.28 ± 0.08	$16.33 \pm 2.05^{***}$
Dry weight shoots (DWS) in gr per plant	2.14 ± 0.29	1.56 ± 0.15	3.06 ± 0.75	3.02 ± 0.06
Dry weight roots (DWR) in gr per plant	0.73 ± 0.02	0.46 ± 0.11	0.76 ± 0.19	1.10 ± 0.14
DWS/DWR	2.92 ± 0.31	2.97 ± 0.7	4.15 ± 0.8	$2.63 \pm 0.19^*$

Values = mean $\pm$ SEM; $n = 3$; simple, double and triple asterisks indicate significant differences respect to the non-stressed control treatment at $P < 0.05$, 0.001 and 0.0001, respectively; Tukey's multiple comparison test.

Table 3
Effect of drought on the photoassimilates distribution of *pRD29A*:oat ADC and wild-type plants.

	Wild type		Lt19	
	Non-stressed	Stressed	Non-stressed	Stressed
Leaf area (cm^2)	$105.5 \pm 11,2$	116.3 ± 14	274.1 ± 46.2	160.1 ± 19.9
LAR (cm^2gr $DW^{-1}_{shoots+roots}$)	36.7 ± 0.05	48.5 ± 9.2	78.6 ± 5.9	$39.3 \pm 5.7^{**}$
SLA (cm^2 gr DW^{-1}_{shoots})	333.8 ± 19.8	401.4 ± 59.1	367.8 ± 14.01	$286.6 \pm 21.8^*$
DW_{shoots}	2.14 ± 0.29	1.56 ± 0.15	3.06 ± 0.75	3.02 ± 0.06
DW_{roots}	0.73 ± 0.02	0.46 ± 0.11	0.76 ± 0.19	1.10 ± 0.14
DW_{shoots}/DW_{roots}	2.92 ± 0.31	2.97 ± 0.7	4.15 ± 0.8	$2.63 \pm 0.19^*$

Values = mean $\pm$ SEM; $n = 3$; simple and double asterisks indicate significant differences respect to the non-stressed control treatment at $P < 0.05$ and 0.001, respectively; Tukey's multiple comparison test.

the proline content. During the stress period, the amount of proline in leaves of *Lt*19 increased significantly relative to that in the irrigated control plants (by a factor of 58). Although to a lesser extent, the proline levels in leaves of the WT plants increased by a factor of 5.3 in relation to the corresponding controls. Furthermore, we observed a redistribution of photoassimilates in *Lt*19 plants that were subject to drought, which changed the ratio between shoots and roots by promoting root growth at the expense of stems and leaves (Table 3). As a result of these morpho-physiological traits of adaptation to drought, the daily consumption of available water for plants of similar ages was significantly lower in the transgenic line (Fig. 1).

3.2. Putrescine accumulation promotes the relative expression of the NCED gene under drought

Considering that the expression of 9-*cis*-epoxycarotenoids dioxygenase (NCED, EC 1.13.11.51), which is a key enzyme that regulates ABA biosynthesis under stress, could be controlled by Put, we determined the enzymatic activity and quantified the final product of the reaction, ABA, in its active and inactive forms.

Fig. 2A shows that the relative expression of the NCED gene was higher in the stressed *Lt*19 lines than in WT plants ($p < 0.0001$), displaying a 10-fold greater expression in response to the water deficit. Furthermore, we observed a positive correlation between the expression of oat ADC and NCED genes ($p = 0.03$), as well as between the contents of free Put and NCED ($p = 0.03$). Although the level of free ABA in shoots slightly increased compared with the WT plants (Fig. 2B), the magnitude of this variation may be attributable to the mechanism of autoregulation of the excess free form by inactivation, primarily through the production of phaseic acid.

Finally, to validate the relation between the accumulation of putrescine and NCED gene expression, a simple experiment was performed in which wild type plants were weekly sprayed with an aqueous solution (100 ppm) of either Put, ABA or tungstate (an inhibitor of ABA biosynthesis) until the soil water potential reached -2 MPa. The endogenous Put content was strongly affected by the phytohormones that were tested. As expected, the level of endogenous free Put was significantly increased by the exogenous application of the phytohormone (Fig. 3A). The foliar spray of ($\pm$) cis, trans-ABA strongly inhibited the accumulation of free Put ($p = 0.01$), which acted as an antagonist of the biosynthesis of PAs.
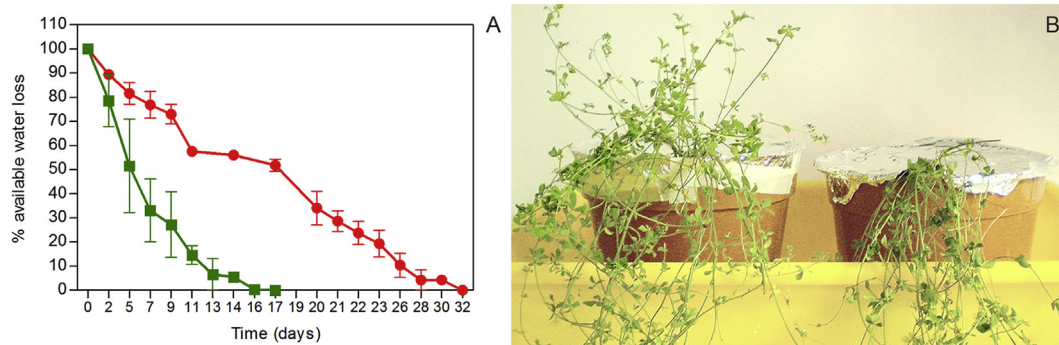

Fig. 1. (A) Water consumption measured as a decrease in available soil water content up to $\psi_{soil} = -2$ MPa. (B) Phenotypic state of transgenic (left) and WTs (right) plants at day 17th of experiment. The values represent the mean ($n = 3$) $\pm$ SEM. Red circles and green square correspond to the *pRD29A*:oat ADC Lt19 line and to WT plants, respectively.
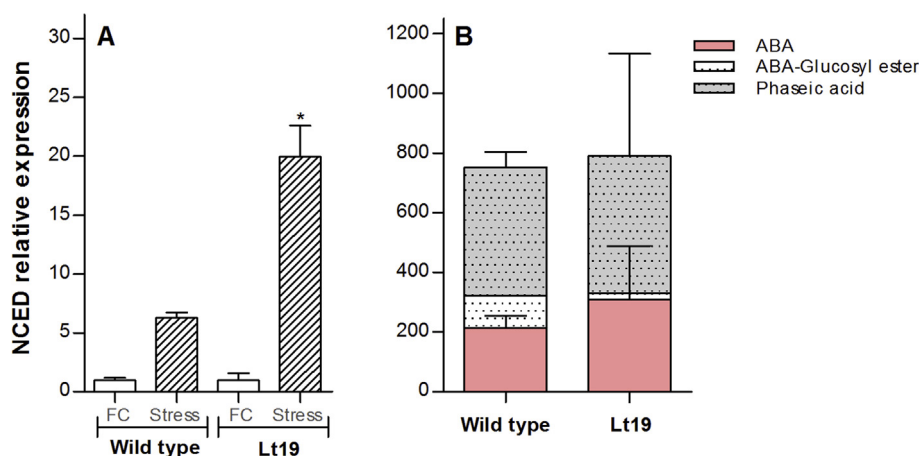
Fig. 2. NCED relative expression (A) and ABA metabolism (B) in the *pRD29A*:oat ADC Lt19 line and in WT plants during dehydration. The values represent the mean ($n = 3$) $\pm$ SEM. Asterisk indicate significant differences respect to the non-stressed control treatment (Tukey's multiple comparison test).

Finally, the endogenous level of Put was restored when the synthesis of ABA was interrupted by tungstate. Fig. 3B shows that NCED gene expression was affected by dehydration, and its transcription was promoted by the exogenous application of Put or ABA and inhibited by tungstate. Additionally, we could detect a slight variation in the free ABA form and in the phaseic acid content as a result of spraying Put (Fig. 3C).

4. Discussion

In this study, we report that a high Put level that was supported by oat ADC overexpression, which was driven by the stress-inducible *pRD29A* gene, improved drought tolerance in *L. tenuis* plants that were grown in soil that was subjected to a gradual decrease in water availability. Our result demonstrated a direct correlation among the increase in *adc* gene expression, enzymatic activity and the accumulation of the final product as Put (Table 1). We have shown that Put accumulation is sufficient to promote drought tolerance because the levels of Spm and Spd did not increase during the dehydration period. Although the intrinsic contributions of Put, Spd and Spm in stress protection remain a matter of analysis, our results are related to those results that were obtained previously in *A. thaliana* that harboured the same construct (Alet et al., 2011). In addition, Alcázar et al. (2010), who studied the response to drought of transgenic *A. thaliana* lines that constitutively expressed the homologous *Arginine*

decarboxylase 2 gene and that contain high levels of Put with no changes in Spd and Spm levels even under dehydration, reported that, at least in this species, the genes that are involved in the synthesis of polyamines are duplicated, and this gene redundancy could be related to certain gene isoforms in the response to specific environmental stimuli.

Additionally, we detected that the osmotic potential in leaves of the transformed line decreased, which indicated a strong osmotic adjustment that was consistent with a significant increase in the proline content in leaves. Proline plays an important role in plant defences against osmotic stresses, and plant with elevated proline levels are known to be particularly tolerant to such stresses (Kishor et al., 2005). The roles of proline and PAs in the drought responses of tobacco plants were recently investigated by Cvikrova et al. (2013) by comparing the responses to drought alone and to drought in combination with heat in leaves and roots of wild type tobacco plants and transformants that constitutively overexpressed a modified gene for the proline biosynthetic enzyme Δ1-pyrroline-5-carboxylate synthetase. Cvikrova et al. concluded that tobacco plants responded to water deficits by accumulating large quantities of proline, particularly in their leaves. The use of an osmotic adjustment mechanism, which is coupled with a reduction in stomatal conductance to counteract the deleterious effects of dehydration and the redistribution of photoassimilates that favour root growth at the expense of the leaf area, strongly suggests that the *pRD29A*::ADC line is more stress-tolerant than the WT plants.
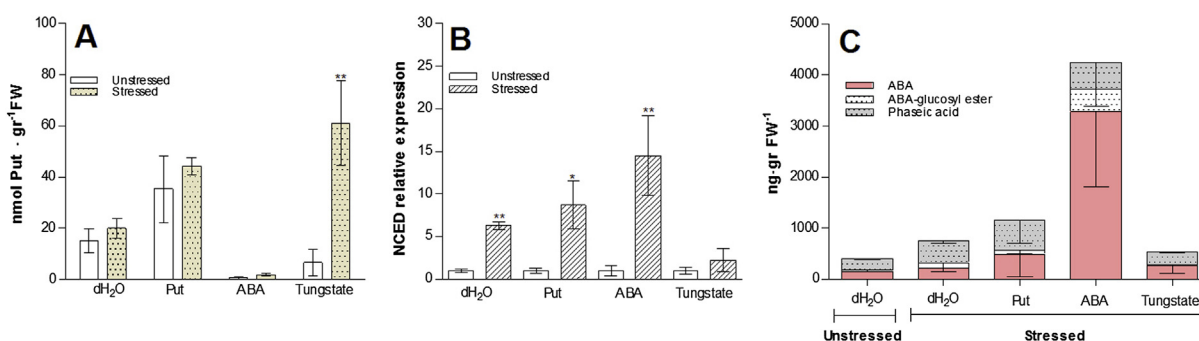

Fig. 3. Non-conjugated putrescine (A), NCED relative expression (B), and ABA metabolism (C) in WT plants that were weekly sprayed with distilled water (dH$_2$O), Put, ABA or tungstate (100 ppm) until the soil water potential reached -2 MPa. The values represent the mean ($n = 3$) $\pm$ SEM. Simple and double asterisks indicate significant differences respect to the non-stressed control treatment at $P < 0.05$ and 0.001 respectively; Tukey's multiple comparison test.

Longer-term physiological responses to abiotic stress conditions are caused by changes in gene regulation through the ABA-mediated regulation of transcription factors that bind to ABA-responsive elements (ABREs) on ABA-regulated genes (Harrison and Khanet al, 2012). Alcázar et al. (2006b) analysed the expression of polyamine biosynthesis genes in *A. thaliana* wild type plants and in mutants that were impaired in ABA biosynthesis (aba2-3) or in signalling (abi1-1) and determined that the *cis* elements ABREs or ABRE-related motifs are found in the promoters region of the *ADC2*, *SPDS1* and *SPMS* genes, which are highly upregulated in response to drought (Bitrián et al., 2012; Alcázar et al., 2006a); simultaneously, both ABA mutants showed much more moderate increases in PAs gene expression. These results evidenced that the transcriptional upregulation of *ADC2*, *SDPS1* and *SPMS* under drought conditions is mediated by ABA, which acts as an upstream regulator of PA biosynthesis in response to drought (Alcázar et al., 2010). Interestingly, the inverse correlation that was observed between non-conjugated Put and ABA contents in *L. tenuis* wild type plants, which was achieved by either the exogenous application of phytohormones or using genetically modified plants with altered levels of PAs, provides evidence that Put acts by inducing the expression of the NCED gene in response to drought. In this way, Cuevas et al. (Cuevas et al., 2008, 2009) performed a detailed integrated analysis of polyamine metabolism under cold stress and, by phenotypical reverse complementation tests for both *adc* and ABA-defective mutants, concluded that putrescine modulates ABA biosynthesis at the transcriptional level in response to low temperatures. Furthermore, Marco et al. (2011) combined the information that was obtained from global transcriptome analyses in transgenic *Arabidopsis* plants with altered Put or Spm levels by the comparison of common and specific gene networks that were affected by elevated levels of endogenous PAs and confirmed that these compounds participate in stress signalling through intricate crosstalk with ABA, Ca^{2+} signalling and other hormonal pathways in plant defences and development.

5. Conclusion

Our results indicate that the overexpression of the oat ADC gene increases the putrescine content in the shoots of *L. tenuis* plants that are subjected to drought. The highest Put content improved the water balance of cells by adjusting the osmotic potential through the release of proline and by adapting the growth pattern of the entire plant by stimulating the development of roots at the expense of the growth of leaves and stems. Additionally, these results suggest that Put controls the level of ABA in response to drought by modulating ABA biosynthesis at the transcriptional level.

Author contributions

E. Espasandin, S. Maiale and P. Calzadilla performed the experiments. O. Ruiz and P. Sansberro designed and instructed the research work. P. Sansberro wrote the manuscript.

Acknowledgements

References

Alcázar, R., García-Martinez, J.L., Cuevas, J.C., Tiburcio, A.F., Altabella, T., 2005. Overexpression of ADC2 in Arabidopsis induces dwarfism and late-flowering through GA deficiency. Plant J. 43, 425—436.

Alcázar, R., Marco, F., Cuevas, J.C., Patron, M., Ferrando, A., Carrasco, P., Tiburcio, A.F., Altabella, T., 2006. Involvement of polyamines in plant response to abiotic stress. Biotech. Lett. 28, 1867—1876.

Alcázar, R., Cuevas, J., Patron, M., Altabella, T., Tiburcio, A.F., 2006. Abscisic acid modulates polyamine metabolism under water stress in Arabidopsis thaliana. Physiol. Plant 128, 448—455.

Alcázar, R., Planas, J., Saxena, T., Zarza, X., Bortolotti, C., Cuevas, J., Bitrián, M., Tiburcio, A.F., Altabella, T., 2010. Putrescine accumulation confers drought tolerance in transgenic Arabidopsis plants overexpressing the homologous Arginine decarboxylase 2 gene. Plant Physiol. Biochem. 48, 547—552.

Alet, A.I., Sanchez, D.H., Cuevas, J.C., del Valle, S., Altabella, T., Tiburcio, A.F., Marco, F., Ferrando, A., Espasandín, F.D., González, M.E., Carrasco, P., Ruiz, O.A., 2011. Putrescine accumulation in Arabidopsis thaliana transgenic lines enhances tolerance to dehydration and freezing stress. Plant Signal. Behav. 6, 1—9.

Bae, H., Kim, S.-H., Kim, M.S., Sicher, R.C., Lary, D., Strem, M.D., Natarajan, S., Bailey, B.A., 2008. The drought response of Theobroma cacao (cacao) and the regulation of genes involved in polyamine biosynthesis by drought and other stresses. Plant Physiol. Biochem. 46, 174—188.

Bitrián, M., Zarza, X., Altabella, T., Tiburcio, A.F., Alcázar, R., 2012. Polyamines under abiotic stress: metabolic crossroads and hormonal crosstalks in Plants. Metabolites 2, 516—528.

Bouchereau, A., Aziz, A., Larher, F., Martin-Tanguy, J., 1999. Polyamines and environmental challenges: recent development. Plant Sci. 140, 103—125.

Bradford, M.M., 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.

Capell, T., Bassie, L., Christou, P., 2004. Modulation of the polyamine biosynthetic pathway in transgenic rice confers tolerance to drought stress. Plant Biol. 101, 9909—9914.

Chiwocha, S.D., Abrams, S.R., Ambrose, S.J., Cutler, A.J., Loewen, M., Ross, A.R., Kermode, A.R., 2003. A method for profiling classes of plant hormones and their metabolites using liquid chromatography-electrospray ionization tandem mass spectrometry: an analysis of hormone regulation of thermodormancy of lettuce (Lactuca sativa L.) seeds. Plant J. 35, 405—417.

Cuevas, J.C., Lopez-Cobollo, R., Alcázar, R., Zarza, X., Koncz, C., Altabella, T., Salinas, J., Tiburcio, A.F., 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.

Cuevas, J.C., Lopez-Cobollo, R., Alcázar, R., Zarza, X., Koncz, C., Altabella, T., Salinas, J., Tiburcio, A.F., Ferrando, A., 2009. Putrescine as a signal to modulate the indispensable ABA increase under cold stress. Plant Signal. Behav. 4, 219—220.

Cvikrová, M., Gemperlová, L., Martinková, O., Vanková, R., 2013. Effect of drought and combined drought and heat stress on polyamine metabolism in proline-over-producing tobacco plants. Plant Physiol. Biochem. 73, 7—15.

Espasandin, F.D., Collavino, M.M., Luna, C.V., Paz, R.C., Tarragó, J.R., Ruiz, O.A., Mroginski, L.A., Sansberro, P.A., 2010. Agrobacterium tumefaciens-mediated transformation of Lotus tenuis and regeneration of transgenic lines. Plant Cell. Tiss. Organ Cult. 102, 181—189.

Garriz, A., Dalmasso, M.C., Marina, M., Rivas, E.I., Ruiz, O.A., Pieckenstain, F.L., 2004. Polyamine metabolism during the germination of Sclerotinia sclerotiorum ascospores and its relation with host infection. New. Phytol. 161, 847—854.

Gill, S.S., Tuteja, N., 2010. Polyamines and abiotic stress tolerance in plants. Plant Signal. Behav. 5, 26—33.

Hao, Y.J., Kitashiba, H., Honda, C., Nada, K., Moriguchi, T., 2005. Expression of arginine decarboxylase and ornithine decarboxylase genes in apple cells and stressed shoots. J. Exp. Bot. 56, 1105—1115.

Harrison, M., 2012. Cross-talk between phytohormone signaling pathways under both optimal and stressful environmental conditions. In: Khan, N.A., et al. (Eds.), Phytohormones and Abiotic Stress Tolerance in Plants. Springer-Verlag, Berlin Heidelberg, pp. 49—76.

Hussain, S.S., Ali, M., Ahmad, M., Siddique, K.H.M., 2011. Polyamines: natural and engineered abiotic and biotic stress tolerance in plants. Biotech. Adv. 29, 300—311.

Kaur-Sawhney, R., Tiburcio, A.F., Altabella, T., Galston, A.W., 2003. Polyamines in plants: an overview. J. Cell. Mol. Biol. 2, 1—12.

Kishor, P.B., Sangam, S., Amrutha, R.N., Sri Laxmi, P., Naidu, K.R., Rao, K.R.S.S., Rao, S., Reddy, K.J., Theriappan, P., Sreenivasulu, N., 2005. Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: its implication in plant growth and abiotic stress tolerance. Curr. Sci. 88, 24—438.

Kuznetsov, V.V., Radyukina, N.L., Shevyakova, N.I., 2006. Polyamines and stress: biological role, metabolism, and regulation. Russ. J. Plant Physiol. 53, 583—604.

Legocka, J., Kluk, A., 2005. Effect of salt and osmotic stress on changes in polyamine content and arginine decarboxylase activity in Lupinus luteus seedlings. J. Plant Physiol. 162, 662—668.

Liu, J.-H., Kitashiba, H., Wang, J., Ban, Y., Moriguchi, T., 2007. Polyamines and their ability to provide environmental stress tolerance to plants. Plant Biotech. 24, 117—126.

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.

Malmberg, R.L., Watson, M.B., Galloway, G.L., Yu, W., 1998. Molecular genetic analyses of plant polyamines. Crit. Rev. Plant Sci. 17, 199–224.

Marco, F., Alcázar, R., Tiburcio, A.F., Carrasco, P., 2011. Interactions between polyamines and abiotic stress pathway responses unraveled by transcriptome analysis of polyamine overproducers. OMICS 15, 775–781.

Nam, K.H., Lee, S.H., Lee, J.H., 1997. Differential expression of ADC mRNA during development and upon acid stress in soybean (*Glycine max*) hypocotyls. Plant Cell. Physiol. 38, 1156–1166.

Peremarti, A., Bassie, L., Yuan, D., Pelacho, A., Christou, P., Capell, T., 2010. Transcriptional regulation of the rice arginine decarboxylase (Adc1) and S-adenosylmethionine decarboxylase (Samdc) genes by methyl jasmonate. Plant Physiol. Biochem. 48, 553–559.

Rakova, N.Y., Romanov, G.A., 2005. Polyamines suppress manifestation of cytokinin primary effects. Russ. J. Plant Physiol. 52, 50–57.

Soyka, S., Heyer, A.G., 1999. Arabidopsis knockout mutation of ADC2 reveals inducibility by osmotic stress. FEBS Lett. 458, 219–223.

Urano, K., Yoshiba, Y., Nanjo, T., Igarashi, T., Seki, M., Sekiguchi, F., Yamaguchi-Shinozaki, K., Shinozaki, K., 2003. Characterization of *Arabidopsis* genes involved in biosynthesis of polyamines in abiotic stress responses and developmental stages. Plant Cell. Environ. 26, 1917–1926.

Wang, J., Sun, P., Chen, C., Wang, Y., Fu, X., Liu, J.H., 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.

Zapata, P.J., Serrano, M., Pretel, M.T., Botella, M.A., 2008. Changes in free polyamine concentration induced by salt stress in seedlings of different species. Plant Growth Regul. 56, 167–177.