RESEARCH PAPER

Plant Signaling & Behavior 6:2, 237-242; February 2011; ©2011 Landes Bioscience

Homeostatic control of polyamine levels under long-term salt stress in Arabidopsis

Changes in putrescine content do not alleviate ionic toxicity

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Key words: arginine decarboxilase, salt acclimation, polyamines, putrescine, spermine, salt overlay sensitive mutants

Abreviations: ADC, arginine decarboxylase; oatADC, arginine decarboxylase from oat (*Oryza sativa*); ABA, abscisic acid; ABRE, ABA-responsive element; DRE, dehydration-responsive element; sos, salt overlay sensitive; WC, water content; RWC, relative water content; RH, relative humidity; SD, standard deviation

Salt stress has been frequently studied in its first osmotic phase. Very often, data regarding the second ionic phase is missing. It has also been suggested that Putrescine or/and Spermine could be responsible for salt resistance. In order to test this hypothesis under long-term salt stress, we obtained *Arabidopsis thaliana* transgenic plants harboring *pRD29A*::oatADC or *pRD29A*::GUS construction. Although Putrescine was the only polyamine significantly increased after salt acclimation in *pRD29A*::oatADC transgenic lines, this rendered in no advantage to this kind of stress. The higher Spermine levels found in WT and transgenic lines when compared to control conditions along with no increment on Putrescine levels in WT plants under salt acclimation, leads us to analyze Spermine effect on *pADC1* and *pADC2* expression. Increasing levels of this polyamine inhibits these promoters expression while enhances *pRD29A* expression, making Spermine the polyamine responsible for salt acclimation, and the transgenic lines developed in this work suitable for studying Putrescine roles in conditions where its biosynthesis would be inhibited in the WT genotype.

Introduction

Salt stress has become a major concern worldwide as salinization of agricultural fields occurs due to irrigation. Some time ago, Munns described the salt stress physiology throughout the two-phase growth model, in which plant physiological responses occur in the period imposed by the two stress components that characterize salinity: hyperosmotic and hyperionic cues.[1] According to this view, salt stress physiology demands the study of ion toxicity rather than osmotic stress, thus implying the need to perform experiments where plants are salt acclimated, instead of salt shocked.[1,2] However, most of the reports rely on experiments where plants are subjected to osmotic stress or salt shock. Although this strategy has rendered amazing molecular breakthroughs (i.e., the ionic regulatory SOS pathway by Zhu[3]), data is lacking concerning signaling under long-term salt stress. On the other hand, polyamines have been associated to abiotic stress response. There are several works that assess for Putrescine as responsible of salt-stress tolerance.[4] Others state that Spermine rather than Putrescine would be involved in this favorable response.[5] In plants, arginine decarboxylase (ADC) is considered to be the rate-limiting step for polyamine biosynthesis under abiotic stress, leading to an increase in Putrescine biosynthesis, a precursor for the higher polyamines Spermidine and Spermine.[4]

We used model plant *Arabidopsis thaliana* and the stress-responsive gene *RD29A* to assess stress signaling in salt acclimated plants. *RD29A* expression is responsive to abscisic acid (ABA), cold and osmotic stress, all of them driven by ABA-dependent and ABA-independent signaling, through ABRE and DRE cis-elements present in the promoter region.[6,7] *RD29A* gene has

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Submitted: 11/12/10; Accepted: 11/16/10
DOI: 10.4161/psb.6.2.14214

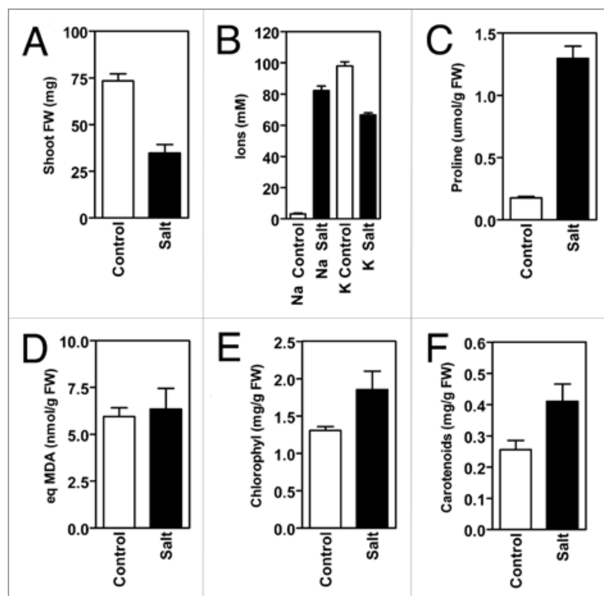

Figure 1. General physiological parameters determined in control (white bars) or salt stress acclimated *A. thaliana* WT plants (final NaCl concentration of 50 mM, black bars). FW: fresh weight, Na: sodium, K: potassium, eq MDA: equivalents of malonaldehide. (A) Plant growth as estimated by shoot fresh weight. Bars represent mean ± SD of three independent experiments, each containing five independent replicates. (B) Sodium and potassium content, expressed as mM in a FW basis. Bars represent mean ± SD of five independent replicates. (C) Proline content. Bars represent mean ± SD of four independent replicates. (E and F) Total chlorophyll (chl a + chl b) and total carotenoid content. Bars represent mean ± SD of six independent replicates. (D) Lipid peroxidation, estimated by TBARs and expressed as equivalents of malonaldehide. Bars represent mean ± SD of four independent replicates. In all cases, salt stress treatment differs statistically to control at $p < 0.05$ according to Student-t test, except in (D).

become a powerful tool for elucidating stress signaling and molecular mechanisms underlying plant responses to stress.[6,8,9] In order to assess the effect of polyamine accumulation on salt-acclimation stress, we obtained transgenic lines of the oat *ADC* under the control of *RD29A* promoter (*pRD29A*::oatADC) in *A. thaliana*.[10] In this work we have integrated the relationship between SOS depletion, response to salt stress and polyamine homeostasis. Finally, we describe the comparative expression of *RD29A*, *ADC1* and *ADC2* promoters under conditions of endogenous Spermine increase, which is a common situation under long-term salt stress.[5,11-13]

Results

Physiological assessment of salt acclimated plants. There are several reports on salt shock but very few on salt acclimation. To get an insight to salt acclimation responses, we have subjected WT and *sos* mutants plants to progressive salt acclimation. *sos* mutants are severely affected by ionic stress and cannot survive at a final concentration of 150 mM NaCl. We therefore used 50 mM NaCl as final concentration for our analyses (**Fig. 1**). As expected, when salinized, growth of WT was reduced almost to a 33% when compared to the control (**Fig. 1A**). Leaf Na^+ content was increased by almost 80% while K^+ content decreased almost to 60% when compared to the control (**Fig. 1B**). Furthermore, the compatible solute proline accumulated seven fold (**Fig. 1C**). No statistically significant increases in lipid peroxidation were observed (**Fig. 1D**), and treated plants were able to complete their life cycle and set seeds even when subjected to a higher salt treatment (150 mM NaCl, final concentration). Chlorophyll content is frequently diminished under salt stress due to stress-induced senescence.[14,15] However, under our salt acclimation conditions we observed a significant increase in chlorophyll and carotene contents in salinized plants (**Fig. 1E and F**), as it has been described in other salt acclimated plant species.[16-18] Taken together, these observations suggest that plants were indeed acclimated to the salt treatment, with no signs of early salt-induced senescence. We did not observe changes in Water Content (WC) under salt stress and Relative Water Content (RWC) decreased 11% (**Table 1**), probably because of salt accumulation and osmotic adjustment. When analyzing ionic content in *sos* mutants, the hypersensitive mutants *sos1-1*, *sos2-2* and *sos3-1* showed a significant Na^+ accumulation (**Fig. 2A**), while no significant change in K^+ accumulation was observed for the last two when compared to WT (**Fig. 2B**) after salt treatment.

When analyzing polyamine content (final concentration 50 mM) WT, *sos2-1* and *sos3-1* mutants did not present changes in free Putrescine content when salt acclimated, although the last ones accumulated statistically more spermidine and spermine than WT in this condition (unpublished results).

Table 1. Water content and relative water content of control and stressed plants

Treatment	WC%	RWC%
Control	94.53 ± 0.13	82.51 ± 0.99
Salt	94.23 ± 0.14	71.87 ± 0.10**

"Salt" refers to salt acclimation treatment (final concentration: 50 mM NaCl). Data are mean ± SE of 4 independent samples, each containing eight rosette leaves of a plant. Statistical differences between treatments and respective non-salinized and salinized controls are shown as: *$p < 0.05$, **$p < 0.01$ (ANOVA-Dunnet's multiple comparison test).

Characterization of the *pRD29A*::oatADC and *pRD29A*::GUS transgenic plants

There are some previous reports suggesting that Putrescine could be responsible for abiotic stress resistance.[19] Since *sos1-1* presented a significant accumulation of Putrescine when subjected to salt stress (resembling the diamine WT accumulation under similar stress conditions), we raised the question whether plants increasing the endogenous Putrescine content upon stress would be capable to survive salt acclimation. Previous reports have provided consistent data demonstrating that *RD29A* gene is transiently responsive to salt stress shock, probably due to osmotic elicitation.[7,8] In order to study the effect of long-term salt stress on *RD29A* expression driving the induction of Putrescine accumulation, we generated *A. thaliana* transgenic lines harboring *pRD29A*::oatADC and *pRD29A*::GUS.

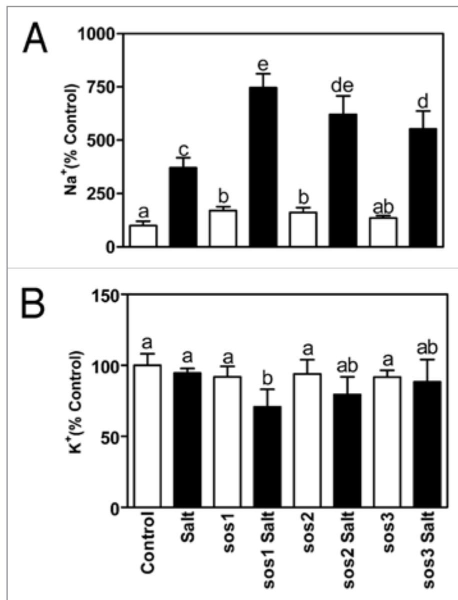

Figure 2. Na^+ and K^+ content in *A. thaliana* WT (Control), *sos1-1*, *sos2-1* and *sos3-1* under non-salinization (white bars) or under salt acclimation (final concentration: 50 mM, black bars). Bars represent media ± SD of sextuplicates. Different letters means values statistically different, according to ANOVA-Bonferroni test ($p < 0.05$).

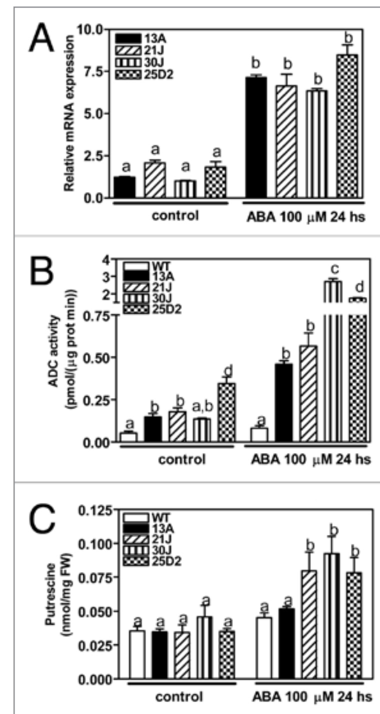

Figure 3. Real-time RT-PCR quantification of *oat ADC* expression (A), ADC activity (B) and free Putrescine content (C) in WT and transgenic plants under ABA treatment. Plants grown in soil for 21 days were harvested after 24 hs of ABA treatment. Control was harvested at the same time than treated plants. No significant difference was observed in *oat ADC* expression (A) inside each group (control or ABA treated). All genotypes but WT presented statistical difference between treated and control ADC activity (B) (data not shown). Different letter means statistical difference inside each group (Control and ABA) according to Two-way ANOVA-Bonferroni test ($p < 0.05$).

A. thaliana pRD29A::GUS plants were treated with ABA and subjected to a β-glucuronidase assay[20] to confirm the activation of the *RD29A* promoter under our experimental growth conditions (data not shown). These experiments confirmed the active induction of *RD29A* promoter in the transgenic lines used in this work, in agreement with previous reports using a heterologous system.[10]

To check the expression of the *oatADC* in *pRD29A*::oatADC lines, transgenic lines were induced with ABA and the transgene expression analyzed by Quantitative-Real Time-PCR (q-rt-PCR) with specific primers. As expected, *oatADC* expression was not detectable in the non-transformed WT whereas in four independent transgenic lines it was highly induced after ABA treatment, and correlated with the induction of ADC activity (**Fig. 3A and B**). Moreover, ABA-induction kinetics of ADC activity in transgenic lines was similar to that described for *RD29A*, whereas there was not significant variation in WT ADC activity under the same conditions (data not shown). No significant differences were found in Putrescine levels between WT and transgenic lines under control conditions (**Fig. 3C**). After ABA induction, at least three of the transgenic lines showed significant Putrescine accumulation (21J, 30J and 25D2, **Fig. 3C**). On the other hand, there were no significant differences on the free contents of Spermidine and Spermine between WT and the transgenic lines under control conditions or after ABA treatment (data not shown).

It is relevant to note that neither *pRD29A*::GUS nor *pRD29A*::oatADC transgenic lines presented phenotypic differences when compared to WT in germination, vegetative growth or development under normal growth conditions (data not shown).

Salt acclimation and differences in *RD29A*, *ADC1* and *ADC2* promoter induction

As expected, free Putrescine content increased almost by 53% in transgenic *pRD29A*::oatADC lines after salt acclimation when compared to WT (**Fig. 4A**). Even though transgenic plants accumulated more Putrescine than WT after treatment, there were no phenotypic differences between them by the end of stress, showing that an inducible Putrescine accumulation is not an advantage under salt acclimation. When looking at the free levels of higher polyamines, we observed an increased level of free Spermine (almost 37%) in WT and transgenic lines after treatment (**Fig. 4B**) with no significant difference between them. In WT, Putrescine levels do not change when Spermine levels do, while in 30J Putrescine and Spermine levels are both increased. We then investigated whether differences between WT and 30J could be due to changes in promoter expression patterns. In fact, *RD29A* promoter expression increased after salt acclimation under our growth conditions almost by 80% (**Fig. 4C**). As an increase in free Spermine levels seems to be a conserved response under long-term salt stress,[5,11-13] we wondered whether the increase in Putrescine level observed in the transgenic line could be due to a positive feed-back effect of Spm over *RD29A* promoter activity.

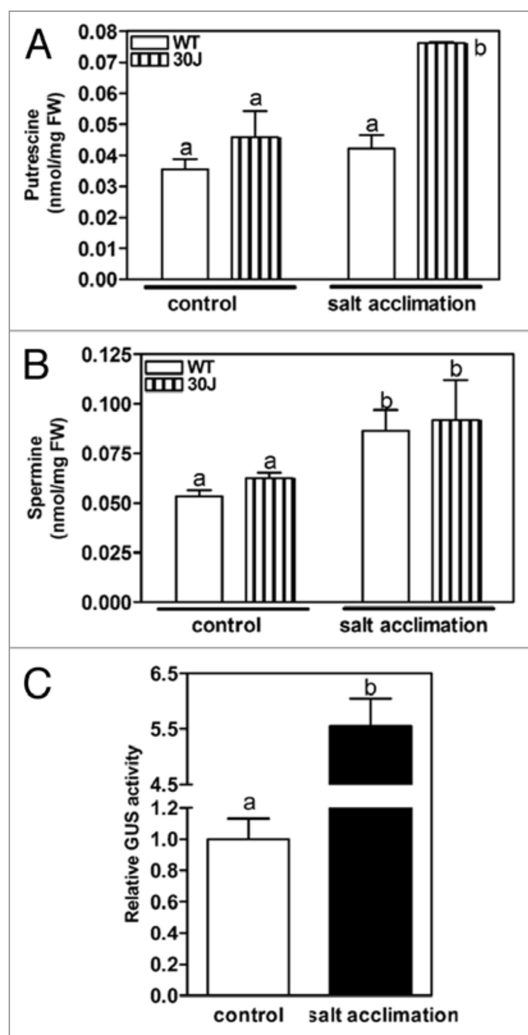

Figure 4. Salt acclimation effect on WT and transgenic lines. WT and transgenic lines were grown in soil. During its life cycle they were subjected to salt acclimation raising the NaCl concentration in the nutrient solution every 3 days (0, 25, 50, 75, 100, 125, 150 mM). After 1 week at 150 mM NaCl, plants were harvested for polyamine quantification (A and B) and GUS activity determination relative to WT (C). Different letter means statistical difference according to t test ($p < 0.05$).

As WT *pADC1* and *pADC2* and transgenic *pRD29A* promoters differ in their cis-regulatory elements (**Fig. 5**), we tested whether Spermine could be activating this pathway in transgenic lines in a way that it could be over-activating *RD29A* expression, being the reason for the higher levels of Putrescine in transgenic lines. Interestingly, while *RD29A* expression increased with higher concentrations of Spermine, both *pADC1* and *pADC2* promoters repressed their expression under the same conditions (**Fig. 6**). We conclude from this assay that the effects of Spm on *RD29A* promoter activity differ from *ADC1* and *ADC2* promoters. That ADC1 and ADC2 promoter activity is downregulated by Spm suggests a negative regulatory feedback loop over Put biosynthesis, which may contribute to polyamine homeostasis under salt acclimation and may represent an advantage for the transgenic plants under a different or consecutive stress.

Discussion

We have described a suitable experimental tool for analyzing the ionic effect of salt stress under long-term salt acclimation. We have also related this type of stress with polyamine responses, stating that Putrescine accumulation is not an advantage under long-term salt stress. There are recent reports that have related Putrescine accumulation to a better tolerance to saline stress.[19] Although there was a higher ADC activity and free Putrescine accumulation in the transgenic lines in this study, there were no phenotypic differences between WT and transgenic lines when salt acclimated. This different response could be due to the different treatment applied. While the long-term salt stress assayed in this study allows us to analyze the ionic effect of salt stress, the previously reported stress resembles a short-term salt stress or "salt shock."[2] Under long-term salt stress, we observed that Spermine content in WT and 30J lines was higher than in the corresponding control condition. This is in agreement with previous reports where it has been shown that Spermine would be involved in a better response of plants to salt stress.[11,12,15] The fact that both WT and transgenic lines accumulated the same level of this polyamine could explain the similar phenotype observed when both lines were salt acclimated.

Simultaneously, we have also described a binary construction harboring an inducible promoter (*pRD29A*::oatADC). This biotechnological approach is useful for the evaluation of Putrescine role in ABA-dependent and ABA-independent stress signaling. In addition, these transgenic lines would be relevant in studying a consecutive stress to salt acclimation, since *RD29A*, *ADC1* and *ADC2* promoters behave in a different way upon high Spermine levels, a polyamine described as the one that accumulates under long-term salt stress. In fact, RD29A expression was induced while ADC1 and ADC2 expression was reduced when Spermine concentration was increased. This result suggests not only a post-translational regulation, as it has been described for oat ADC,[21] but also a transcriptional regulation. The inhibition in ADC1 and ADC2 expression could be due to a negative feedback loop, which makes sense if we take into account that Spermine is toxic for plants at high concentrations levels.[22] Further studies are needed to assess a potential retro-regulatory response.

Materials and Methods

Plant material, growth conditions and treatments. Abdelhak El Amrani and the Arabidopsis Biological Resource Center (Ohio State University, Columbus) kindly provided *A. thaliana pADC1*::GUS and *pADC2*::GUS[23] and Col-0 gl1 and *sos* mutants seeds respectively. Since all *sos* mutants, oat ADC and GUS transgenic plants were *gl*1 background, we utilized *gl1* mutant as control and is termed as WT in this work to simplify nomenclature. In our lab we utilized binary vectors harboring GUS- or oatADC-encoding sequences under the control of the stress-inducible promoter *RD29A*[10] to transform *A. thaliana* Col-0 *gl1* plants by *floral dip*. The seeds selection was performed in a medium MS with Kanamycine until reached T3. For experimental work, we selected four independent lines harboring the *pRD29A*::oatADC

construction (13A, 21J, 30J and 25D2) and one carrying *pRD29A*::GUS (21C). By PCR and Southern blot analysis we confirmed transgenic status. After that, stratified seeds were sown in pots filled with soil:sand:perlite (2:1:1). Plants were sub-irrigated with half-strength Hoagland's nutrient solution and grown in a growth room with a 16/8 h photoperiod at 24 ± 2°C, 50/75 ± 5% RH (day/night) and a photon flux density of 100 μmolm^{-2}s^{-1}. Salt stress treatments started 4 days after imbibitions and lasted 20 days, before flowering. Acclimation to salinity was achieved by increasing the NaCl content of the nutrient solution every 4 days in three consecutive steps (10, 25 and 50 mM NaCl), thus plants stayed at least 8 days at the final NaCl concentration. Where indicated, a more drastic salinization experiment was performed, which consisted in increasing NaCl content of the nutrient solution every 2 days to a final concentration of 150 mM in 6 consecutive steps (25, 50, 75 100, 125 and 150 mM NaCl). Therefore, plants stayed at least 6 days at 150 mM NaCl. Further treatments were performed along with non-salinized controls once the whole salinization period was completed, and plant material sampled at midday. "Spermine stress" was performed germinating and growing the seedlings 11 days in MS medium supplemented with different Spermine concentrations (0, 50, 250 and 500 μM). ABA treatment consisted in the spraying of a 100 μM ABA (Sigma) solution on 3-week-old plants (before bolting) 24 h before sampling. In all cases, experiments were repeated at least twice, and representative experiments are shown.

Physiological and analytical determinations. Percentages of Water Content (WC) and Relative Water Content (RWC) were used as general physiological markers of water balance status (Koide et al. 1992).[30] Photosynthetic pigments were determined as described by Lichtenthaler.[24] Proline content was determined by ninhydrin reaction under conditions described in reference 11, and lipid peroxidation was estimated spectrophotometrically as thiobarbituric acid reactive substances (TBARs) according to Hodges et al.[25] We estimated the levels of Na^+ and K^+ by standard flame photometry. GUS activity was determined as described by Jefferson,[20] and normalized to the corresponding controls (see figure legends). We determined the protein content by Bradford method using bovine serum albumin as the reference standard.[26]

Quantitative RT-PCR. We performed the quantitative RT-PCR of *oatADC* expression analysis on the ABIPrism® 7000 Sequence Detection System (Applied Biosystems), and Power SYBR® Green PCR Master Mix (Applied Biosystems) was used to amplify and quantify the cDNA. As internal control, we utilized the gen Ubiquitin-10 (*UBQ-10*; At4g05320). The PCR conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 30 s and 60°C for 1 min. Three replications performed for each sample in each experiment were developed. The melting curves were obtained with help of the Dissociation Curves® software (Applied Biosystems) to ensure the amplification of the only one product. Analysis of gene expression data was done with the $2^{-\Delta\Delta Ct}$ method[27] using the DART-PCR Version 1.0 datasheet as described by Peirson et al. Peirson et al. described similar amplification efficiency.

PCR primers were oatADC F: 5'-AGT TAC GAC GTG AAA CAG GAT ATC A-3' and R: 5'-CCA CCA TTT CCC ACA CCT TA-3', UBQ-10 (At4g05320) F: 5'-TAA TCC CTG ATG AAT AAG TGT TCT AC-3' and R: 5'-AAA ACG AAG CGA TGA TAA AGA AG-3'.

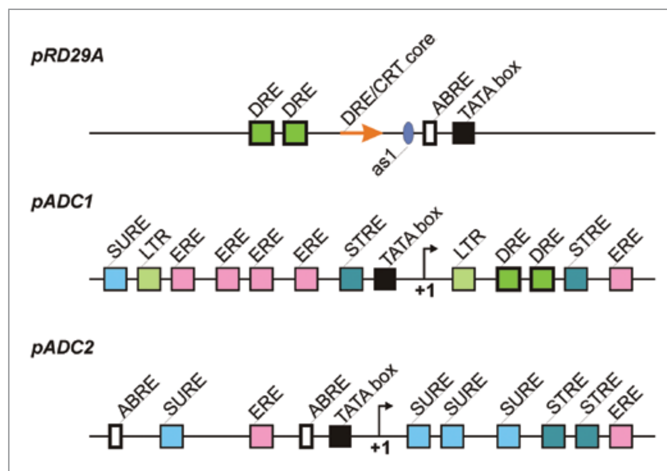

Figure 5. cis-elements in the *RD29A*, *ADC1* and *ADC2* promoters. +1 indicates the transcription start site and the regulatory sequence UTR 5'. *pRD29A*: promoter for *RD29A*; *pADC1*: promoter for *ADC1*; *pADC2*: promoter for *ADC2*. SURE: sucrose-responsive element. LTR: low-temperature responsive element. ERE: ethylene-responsive element. STRE: stress-responsive element. DRE: dehydration-responsive element. ABRE: ABA-responsive element. as1: cis-element involved in root expression on oxidative stress. (Adapted from ref. 6, 23 and 29).

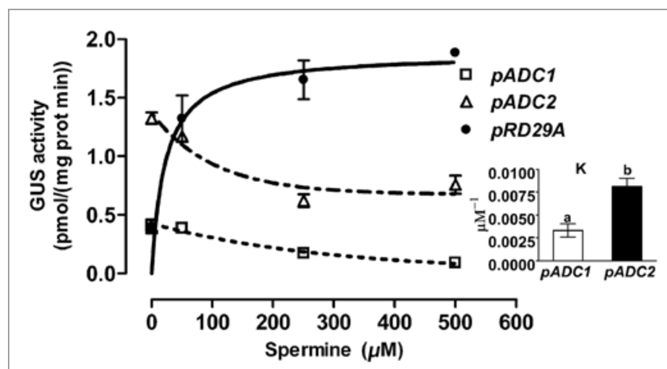

Figure 6. *RD29A*, *ADC1* and *ADC2* promoter expression under Spermine treatment. *pRD29A*::GUS, *pADC1*::GUS and *pADC2*::GUS transgenic lines were germinated and grown in MS plates supplemented with 0, 50, 250 and 500 μM Spermine. GUS activity was determined 11 days post-imbibition. Infograph represents decay constant K for *pADC1* and *pADC2* expression. Different letter means statistical difference according to t-test ($p < 0.05$).

Statistical analysis. Student t test and ANOVA analysis were performed to asses statistical differences among treatments, using the GraphPad Prizm 4.0 software.

Acknowledgements

We are most grateful to Dr. Abdelhak El Amrani for kindly providing the *A. thaliana pADC1*::GUS and *pADC2*::GUS seeds. We want to thank Dr. Ricardo Di Masso (Cátedra de Genética

Experimental, Facultad de Medicina, Universidad Nacional de Rosario) for his invaluable assistance on statistical analysis. This work was supported by grants PICT of Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT, Argentina), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina), San Martin University and AECI (Spanish International Cooperation Agency) to OAR and grants BIO2005-09252-C02-01 and BIO2008-05493-C02-01 to A.F.T. A.F.T., T.A., P.C. and O.A.R. also acknowledge grants-in-aid from COST-Action FA0605. O.A.R. is member of the research committee from CONICET.

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