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Down-regulation of arginine decarboxylase gene-expression results in reactive oxygen species accumulation in Arabidopsis

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Abstract

Arabidopsis amiR:*ADC*-L2 is a non-lethal line with several developmental defects, it is characterized by a drastic reduction in free polyamine content. Herein, we found that catalase application had growth promoting effects in amiR:*ADC*-L2 and parental Ws seedlings. Differences in ROS content between amiR:*ADC*-L2 and Ws seedlings were detected. Increased H_2O_2 levels were found in the amiR:*ADC*-L2, as well as low *AtCAT2* gene expression and reduced catalase activity. Estimation of polyamine oxidase activity in amiR:*ADC*-L2 line indicated that the over-accumulation of H_2O_2 is independent of polyamine catabolism. However, increments in NADPH oxidase activity and $O_2^{\bullet -}$ content could be associated to the higher H_2O_2 levels in the amiR:*ADC*-L2 line. Our data suggest that low polyamine levels in Arabidopsis seedlings are responsible for the accumulation of ROS, by altering the activities of enzymes involved in ROS production and detoxification.

Keywords: Arabidopsis; arginine decarboxylase; polyamines; reactive oxygen species; catalase activity.

1. **Introduction**

Polyamines (PAs) are nitrogenous compounds essential for living organisms. The most abundant PAs are diamine putrescine (Put), triamine spermidine (Spd), tetramines spermine (Spm), and thermospermine (Tspm). PAs play an important role in plant growth and development, and in biotic and abiotic stress responses [1].

Reactive oxygen species (ROS) are generated during plant development as products of metabolic processes and in response to diverse stress conditions. An important connection between PA and ROS has been reported [2]. It has been suggested that PAs play a dual role on the redox status of the cell. For instance, PAs participate on the oxidative burst mitigation caused by ROS accumulation [3,4]. Besides, H_2O_2 production and accumulation can be the result of PA catabolism by amine oxidase (DAO; EC 1.4.3.6) and polyamine oxidase (PAO; EC 1.5.3.3) enzymes [5]. In biological systems, H_2O_2 acts as a signaling molecule that regulates gene expression with effects at the cellular, physiological and developmental level [6]. However, under stress conditions over-accumulation of H_2O_2 and other ROS might result in several negative effects in plant cells such as the disruption of photosynthetic efficiency, chloroplasts clustering, lack of mitochondrial potential, and cytotoxic damage by the oxidation of lipids, proteins and DNA [7,8,9,10]. The relationship between positive effects and cell deterioration caused by ROS will depend on its concentration, cell localization and transport [11].

In *Arabidopsis thaliana*, the arginine decarboxylases ADC1 and ADC2 are the unique enzymes involved in Put biosynthesis [1]. Our group reported an Arabidopsis transgenic line (amiR:*ADC*-L2) that succeeded to decrease *ADC* gene expression by the down-regulation of *ADC* paralogues [1]. The silenced amiR:*ADC*-L2 line had very low PA levels, even lower than each *adc1* or *adc2* single insertional mutants, and exhibited defects in growth and development [1]. In this study, the effect of exogenous catalase (CAT) application on the amiR:*ADC*-L2 growth was evaluated. ROS levels and enzymatic activities of CAT, PAO and NADPH oxidases were determined in the amiR:*ADC*-L2 line. Our results indicate that the amiR:*ADC*-L2 plantlets with low PA levels have alterations in $O_2^{\bullet -}$ and H_2O_2 contents, an aspect that can be attributed to deregulation of NADPH oxidase and CAT activities.

2. Materials and Methods

2.1 Plant material and growth conditions

Seeds of *A. thaliana* ecotype Wassilewskija (Ws) and amiR:*ADC*-L2 line were used [1]. The seeds were surface-sterilized with a chlorine solution 20% (v/v) for 7 min followed by four rinses with sterile distilled water. Subsequently, the seeds were stratified at 4°C, 48h in the dark. Afterwards, seeds were germinated and grown on Petri plates with Murashige and Skoog medium (MS) 0.5x (pH 5.7) containing 0.5% (w/v) sucrose, and 0.8% (w/v) phytagel. Plates were incubated at 22±2°C in a growth chamber with a photoperiod of 16h light (100µmol m^{-2} s^{-1}) and 8h dark.

2.2 Estimation of plant fresh weight, primary root length and chlorophyll content

Seeds from amiR:*ADC*-L2 and Ws ecotype were grown on MS for seven days. Seedlings were transferred to MS plates containing 0, 200, and 300 U/mL catalase (CAT). Each treatment was maintained in a growth chamber for five days under photoperiods of 16h light (100µmol m^{-2} s^{-1}) and 8h dark at 22±2ºC. Fresh weight (mg) and primary root length (cm) were measured. The values obtained represent the means from 15 seedlings of each line (*n=15*). The image analysis software IMAGE J (http://rsb.info.nih.gov/ij) was used to evaluate the primary root length. Chlorophyll extraction was done by incubation of seedlings in 0.7 mL *N,N*-dimethylformamide for 24h in the dark and continuous agitation; and spectrophotometric estimation of chlorophyll (µg/g FW) at 647 and 664 nm was carried out.

2.3 Reactive oxygen species (ROS) detection by fluorescence microscopy

ROS production was visualized *in planta* by the use of the fluorescent molecule 2’,7’-dichlorodihydrofluorescein diacetate (DCFH2-DA), as is described by [12], with some modifications. Seeds of the amiR:*ADC*-L2 line and Ws ecotype were germinated and grown *in vitro* in 0.5x MS petri plates under control conditions during ten days. Then, the seedlings were transferred to 24-well culture plates containing MS liquid medium supplemented with: 200U/mL CAT, 10µM Put, 10µM Spd, 10µM Spm, 50µM Put, 50µM Spd, and 50µM Spm. Seedlings were observed under a fluorescence microscope (Zeiss Axio Imager M2/ 3525000525) using an excitation and emission wavelength of 480 nm and 500-550 nm, respectively. At least, six seedlings of each line were analyzed per treatment. Each assay was repeated three times.

2.4 Hydrogen peroxide and superoxide radical anion quantification

The H_2O_2 and $O_2^{\bullet-}$ contents in the amiR:*ADC*-L2 line and Ws were estimated in ten-day-old seedlings. H_2O_2 quantification (µmol g^{-1} FW) was determined from 100 mg of fresh plant material using potassium iodide (KI), as is described by [13]. The $O_2^{\bullet-}$ quantification was performed using 200 mg of fresh vegetal tissue in accordance to [14].

2.5 Hydrogen peroxide histochemical staining

In situ H_2O_2 detection was performed in 12-day-old seedlings (amiR:*ADC*-L2 line and Ws) grown under control conditions using 3,3-diaminobenzidine (DAB) staining (Sigma-Aldrich) as reported by [15]. Ascorbic acid (10 mM) treatments were used as negative controls. Samples were observed under the stereoscope (Motic SMZ-143). Ten seedlings of each line were analyzed by treatment. Assays were repeated three times.

2.6 RNA isolation and qRT-PCR analysis

Total RNA was isolated from ten-day-old Ws and amiR:*ADC*-L2 seedlings using Concert reagent (Invitrogen) according to the manufacturer's instructions, followed by DNAse Turbo digestion (Ambion). qRT-PCR analysis was performed as previously described [16]. The *A. thaliana* ubiquitin 5 (*UBQ5*) was used as reference gene. Relative expression levels of *AtCAT2* gene was presented as $2^{-\Delta Ct}$, where $\Delta Ct = Ct_{AtCAT2} - Ct_{UBQ5}$ [17]. The following oligonucleotides were used: *AtCAT2*-FW (5’-TACCGTACCTTTACACCAGAGA-3’), *AtCAT2*-RV (5’-TCTGTCCCAAAGACTTATCAGC-3’), *AtUBQ5*-FW (5’-TCGACGCTTCATCTCGTCCT-3’) and *AtUBQ5*-RV (5’-CGCTGAACCTTTCCAGATCC-3’).

2.7 Catalase activity

Catalase enzymatic activity in the amiR:*ADC*-L2 line and the Ws ecotype was estimated in ten-day-old seedlings according to [18], with some modifications. With this purpose, seeds were germinated on a mesh placed on top of 0.5x MS medium and grown for eight days under controlled conditions. Seedlings were then transferred on the mesh to fresh medium containing 0 or 10 µM Put for 48 h. Catalase activity was measured as decrease in H_2O_2 absorbance at 240 nm after one minute of reaction. The molar extinction coefficient of H_2O_2 at 240 nm (43.6 $M^{-1}cm^{-1}$) was used for CAT activity estimation. Enzymatic activity was expressed as U/mg of protein.

2.8 PAO enzymatic activity

Protein extraction was done using 200mg of ten-day-old Ws and amiR:*ADC*-L2 seedlings. Samples were homogenized in 400µl of 200mM sodium phosphate buffer (pH 7.0; tissue to buffer ratio 1:2, w/v) containing 1mM PMSF. Total protein content was measured by [19]. PAO activity (nKat/mg protein) determination was performed following standard protocols [20].

2.9 NADPH oxidase activity

Seeds of Ws and amiR:*ADC*-L2 were germinated on plates with MS medium and grown for eight days under controlled conditions. Subsequently, seedlings were transferred to fresh medium containing 0 or 10 µM Put for 48 h. Assays were made from 200 mg of vegetal tissue. Total protein concentration was estimated by the Bradford method [19]. NADPH oxidase activity was determined as described by [21]. Polyacrylamide gels were scanned with the Pharos FX system (Biorad) and NTB band intensity was detected with the Image J program. NADPH oxidase activity was expressed as relative units (RU).

2.10 Statistical analysis

Two-way analysis of variance (ANOVA) and Bonferroni test analyses were performed to assess statistical significance. The GraphPad Prism version 5.0b (GraphPad Software, San Diego, California, USA) was used. Data represent the mean ± SE. Differences at $P < 0.05$ were considered significant.

3. Results

3.1 Catalase application had growth promoting effects in amiR:*ADC*-L2 and Ws seedlings

The effect of exogenous CAT application on the amiR:*ADC*-L2 line [1] and parental Ws was assessed, and data on fresh weight, chlorophyll content, primary root length and the number of lateral roots in 12 day-old-seedlings were collected and analyzed. A growth promoting effect in both amiR:*ADC*-L2 and Ws seedlings was observed with dose-dependent CAT application, inducing an increase in fresh weight, root length, and chlorophyll content (Figures 1A-D). The amiR:*ADC*-L2 line had an increased number of lateral roots in comparison to the parental line under control growth conditions (Fig. 1E; [1]); however, under CAT treatments the number of lateral roots in amiR:*ADC*-L2 were similar to the observed in parental (Figure 1E).

3.2 The content of reactive oxygen species was increased in the amiR:ADC-L2 line

The growth promoting effect of CAT application in amiR:*ADC*-L2 and Ws suggested that ROS content (mainly H_2O_2) could be a limiting factor for Arabidopsis growth. We detected ROS in root tips of Ws and amiR:*ADC*-L2 seedlings using DCFH2-DA dye. Under control growth conditions, ROS signal was clearly perceived in the amiR:*ADC*-L2 line as green fluorescence in contrast to Ws, in which no signal was detected (Figure 2A). When CAT was applied, ROS signal was completely abated in amiR:*ADC*-L2 (Figure 2A). We observed that the supplementation of each PA (10 µM Put, Spd or Spm) abated the green fluorescence signal associated to ROS in the silenced line, which reinforced the importance of PAs in ROS homeostasis (Figure 2B). However, Ws seedlings exhibited marked ROS signal under higher PA concentrations (10 and 50 µM), this observation could be the result of PA catabolism in a genetic background with normal PA content. In the amiR:*ADC*-L2 line a similar behavior was observed only a the highest PA concentration tested (50 µM of each PA; Figure 2C), suggesting that the excess of incorporated PA was metabolized (i.e. degraded by amine oxidases).

I*n situ* H_2O_2 production in different tissues of amiR:*ADC*-L2 line was determined by DAB staining (Figure 3A). A higher accumulation of H_2O_2 was observed in the shoot apex, cotyledons, hypocotyl and the root tip. These data indicate that the H_2O_2 accumulation in amiR:*ADC*-L2 line is not restricted to root tip zone. Furthermore, quantitative estimation of H_2O_2 showed that the amiR:*ADC*-L2 line had 28.2% higher H_2O_2 content than Ws seedlings (Figure 3B).

3.3 *AtCAT2* expression level and CAT enzymatic activity were down-regulated in the amiR:*ADC*-L2 seedlings

In order to explore whether the decrease in PA levels affects components of the antioxidant system, *AtCAT2* gene expression levels and CAT activity were estimated. A 40% reduction in *AtCAT2* expression levels, and a 27% reduction in CAT activity were observed in the amiR:*ADC*-L2 line in comparison to Ws seedlings (Figure 4A). Interestingly, when amiR:*ADC*-L2 was treated with 10 μM Put for 48h, an increment in CAT activity was achieved in comparison to Ws (Figure 4B). These data suggest that H_2O_2 accumulation in the amiR:*ADC*-L2 line, which has reduced PA content, could be associated with a decrease in *AtCAT2* expression and CAT activity.

3.4 The amiR:*ADC*-L2 seedlings showed no changes in PAO activity

Changes in ROS content in the amiR:*ADC*-L2 line could also be attributed to changes in PA oxidation activity. Therefore, we determined PAO activity in amiR:*ADC*-L2 and Ws seedlings supplemented with 0 or 10 μM Put. No significant differences in the Spd and Spm oxidation activities between the amiR:*ADC*-L2 and Ws seedlings were detected (Figures 4C and D). These data suggest that H_2O_2 accumulation in the amiR:*ADC*-L2 line is not related PA catabolism.

3.5 The $O_2^{\bullet -}$ content and NADPH-oxidase activity were increased in amiR:ADC-L2

Owing H_2O_2 accumulation in amiR:*ADC*-L2 line, we asked if $O_2^{\bullet -}$ levels were also increased. A significant increase in $O_2^{\bullet -}$ content was noticed in the amiR:*ADC*-L2 (52%) line with respect to Ws (Figure 4E). This observation was in accordance with a significant increase (81%) in NADPH activity in amiR:*ADC*-L2. Conversely, in the presence of Put a reduction in the $O_2^{\bullet -}$ levels was noticed in the silenced line as well as a decrease in NADPH activity, achieving a similar NADPH activity as in Ws (Figure 4F).

4. **Discussion**

Exogenous application of PAs improves plant stress tolerance and promotes plant growth [22, 23]. In contrast, suboptimum levels of PAs are related to stress susceptibility and developmental disturbance [1, 16]. Herein, we further characterized the amiR:*ADC*-L2 line evaluating some physiological parameters associated to growth and ROS production. Decline in chlorophyll levels was observed in amiR:*ADC*-L2 line; this aspect could be related to the low PA content, and the role of PAs in chloroplast. It has been reported that ADC proteins are located mainly in chloroplasts [24]. Kasinathan and Wingler [25] reported that mutant lines with lower ADC activity had decreased chlorophyll content and reduced photosynthetic efficiency. These observations are in accordance with the functions attributed to PAs in the protection of the photosynthetic apparatus, avoidance of chlorophyll loss, inhibition of thylakoid destruction, protection of PSII proteins and improved photochemical efficiency under stress conditions [5, 26].

We analyzed the effect of CAT application on amiR:*ADC*-L2 plantlets. We obtained an improved growth of amiR:*ADC*-L2 under CAT application, with a dose-dependent effect, as reflected by increments in fresh weight, primary root length and chlorophyll content. However, the stimulant effect was also observed in Ws. The beneficial effect of CAT application on both lines suggests ROS content could be a limiting factor for Arabidopsis growth in an *in vitro* culture system. *CAT2* gene expression and CAT activity were found down-regulated in the amiR:*ADC*-L2 line. Arabidopsis genome contains three CAT genes. Among them, *CAT2* is one of the major isoforms in rosette tissues, with important functions in ROS detoxification. *Atcat2-1* mutants had 90% decrease in CAT activity in leaves, and manifested dwarf phenotypes at growth irradiances of 50-100 μmol m^{-2} s^{-1} or higher, a phenotype that was linked with photorespiratory ROS production [27]. As well, Du et al., [28] indicated that reduction of CAT activity in Arabidopsis *cat2-1* mutant line conduced to H_2O_2 accumulation under normal growth conditions. The amiR:*ADC*-L2 line showed stunted growth, an aspect initially attributed to the lack of PAs, but the observation of increased H_2O_2 and $O_2^{\bullet-}$ levels, and alteration in CAT activity let us suggest that changes in the antioxidant capacity of the silenced line could also be responsible for phenotypic alterations. Recently, Fu and collaborators [29] reported that *AtADC2* overexpression line (AtADC2-OE) was more tolerant to salt stress, it had reduced H_2O_2 levels and enhanced CAT activity, reinforcing that Put accumulation is required for the regulation of antioxidant enzymes activities and for salt stress tolerance [29].

Application of 10 μM PAs alleviated the accumulation of H_2O_2 in amiR:*ADC*-L2 root tips, supporting the idea that low PA levels alter ROS homeostasis. In Ws seedlings addition of 10 and 50 μM PAs lead to an increment in H_2O_2 signal in root tips. Likewise, at higher PA concentration (50 μM) the amiR:*ADC*-L2 augmented fluorescence signal. This phenomenon could be related to the catabolism of the exogenous added PAs and the concomitant production of H_2O_2 [30]. Several studies have reported that PAs can modulate H_2O_2 levels in plants [31]. It has been reported that H_2O_2 production at low levels through PA catabolism, contributes to plant growth.

However, the over-accumulation of H_2O_2 can be toxic for the plant and lead to growth arrest; at this phase of stress PAs can act as chelators of ROS [32, 33]. Put triggers the antioxidant system by inducing antioxidant enzymes and increasing the content of non-enzymatic antioxidants [34, 35]. Verma and Mishra [36] described that Put application in *Brassica juncea* mitigated the negative effects caused by salinity. Put treatment increased the activity of different antioxidant enzymes including CAT. In this regard, we reverted the low CAT activity observed in amiR:*ADC*-L2 line with Put application, and we confirmed that total PAO activity showed no changes in the amiR:*ADC*-L2 mutant. Finally, the augmented NADPH oxidase activity in amiR:*ADC*-L2 suggested that H_2O_2 accumulation could be the result of a major production of $O_2^{\bullet -}$, and perhaps this phenomenon could be dependent on PA levels. In agreement, Papadakis and Roubelakis [37] reported that exogenous Put application could inhibit the NADPH oxidase activity by concentration dependent way in protoplast cells of *Nicotiana tabacum.* Our data reinforce that PAs are modulators of ROS. Low PA levels in amiR:*ADC*-L2 could be responsible for ROS accumulation as a consequence of altered NADPH oxidase and CAT activities.

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Conflicts of Interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1. Effect of catalase application in the Arabidopsis amiR:*ADC*-L2 and Ws. A) Phenotype of 12-day-old amiR:*ADC*-L2 and Ws seedlings grown in 0.5x MS plates supplemented with 0, 200, and 300 U/mL CAT. The scale bar corresponds to 1 cm. Measurement of fresh weight (B), total chlorophyll (C), primary root length (D) and number of lateral roots (E) were determined and represented graphically. The average fresh weight and primary root length are presented as milligrams per plant and cm, respectively. Data are means ± SE from three biological replicates ($n=15$). Total chlorophyll was expressed as µg per g FW, and data are means ± SE from five biological replicates ($n=5$). Letters indicate a significant difference according to Two-way ANOVA analysis ($P>0.05$).

Figure 2. Detection of DCFH2-DA green fluorescence in Arabidopsis root tips of WS and amiR:*ADC-L2* line. Ten-days-old seedlings were incubated 4 h in: (A) 0.5x MS liquid medium (un-treated), MS + 200U/mL CAT, (B) MS plus 10 µM Put, 10 µM Spd or 10 µM Spm, (C) MS plus 50 µM Put, 50 µM Spd or 50 µM Spm. Two representative microscopy images of DCFH2-DA fluorescence (at 10x magnification) in each treatment are shown.

Figure 3. Determination of H_2O_2 content in Arabidopsis amiR:*ADC*-L2 and Ws. (A) Histochemical detection of H_2O_2 by DAB staining in amiR:*ADC*-L2 and Ws seedlings. Control staining's were performed in the presence of 10 mM ascorbic acid (AsA). (B) H_2O_2 quantification was performed using KI in amiR:*ADC*-L2 and Ws seedlings. Data are means ± SE from three biological replicates ($n=3$). The asterisk indicates significant differences between Ws and amiR:*ADC*-L2 lines by unpaired *t*-test ($P<0.05$).

Figure 4. CAT, PAO and NADPH enzymatic activities. Ten-day-old amiR:*ADC*-L2 and Ws seedlings grown in MS 0.5x were analyzed. (A) Transcript levels of *AtCAT2* were estimated by the qRT-PCR approach, using the *A. thaliana UBQ5* as reference gene. (B) Estimation of CAT activity in plants treated with 0 or 10 µM Put for 48 h. (C and D) Estimation of PAO activity, as substrates 2 µM Spd or Spm were used. (E) Estimation of $O_2^{\bullet -}$ content and (F) NADPH oxidase activity in seedlings treated with 0 or 10 µM Put for 48 h. Data are means ± SE from three biological replicates ($n=3$). The asterisks indicate significant differences between Ws and amiR:*ADC*-L2 un-treated and under Put treatment using unpaired *t*-test ($P<0.05$).