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Research article

Allantoin contributes to the stress response in cadmium-treated Arabidopsis roots

Maryam Nourimand, Christopher D. Todd*

Department of Biology, University of Saskatchewan, Saskatoon, S7N 5E2, Canada

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ABSTRACT

Ureides are nitrogen-rich compounds, derived from purine catabolism. A dual role for ureides, and for allantoin in particular, in both nitrogen recycling and the abiotic stress response has been recently identified. Previous work on the effect of allantoin on cadmium (Cd)-exposed Arabidopsis revealed that high concentration of allantoin in allantoinase-negative mutant (*aln-3*) leaves alleviates Cd toxicity via inducing antioxidant mechanisms in these plants. In the present study, we evaluate whether allantoin has a similar protective role in roots. Both wild type and *aln-3* roots contain higher amounts of internal Cd compared to leaves. Likewise, *aln-3* roots are more resistant to Cd, reflected in fresh and dry weight, and stimulated antioxidant enzyme activity, including superoxide dismutase (SOD) and catalase (CAT), resulting in lower reactive oxygen species concentration. In contrast with wild-type leaves, high levels of Cd in Col-0 roots reduces transcript abundance of uricase, leading to a significant decline in allantoin level of treated roots at 1000 and 1500 μM $CdCl_2$. This metabolite change is also accompanied by decreasing the activity of antioxidant enzymes (SOD and CAT). Additionally, contrary to wild-type leaves, root genotype has a significant effect on CAT activity under Cd treatment, suggesting the possible different sources of damage and oxidative stress response in these two tissues.

1. Introduction

Ureides are nitrogen rich compounds derived from purine rings. In plants the catabolism of purines, primarily from nucleic acids, results in xanthosine production which generates xanthine, the first compound of ureide pathway (Alamillo et al., 2010; Coleto et al., 2014; Diaz-Leal et al., 2012). What is commonly referred to as the ureide pathway starts with the conversion of xanthine to uric acid and proceeds through a series of enzymatic reactions, releasing nitrogen in the form of ammonia (NH_4^+) (Supplementary Fig. S1). This process has an important role in nitrogen transport, storage, and recycling in plants under different developmental and physiological conditions (Alamillo et al., 2010; Zrenner et al., 2006).

Recent studies have proposed that ureide metabolism is not only associated with nitrogen remobilization, but also with the abiotic stress response. Two ureides, allantoin and allantoate, have been considered as potential reactive oxygen species (ROS) scavengers, participating in the plant stress response and conferring tolerance to abiotic stressors (Takagi et al., 2016; Watanabe et al., 2014). Accumulation of allantoin and allantoate in *Arabidopsis thaliana* in response to dark treatment results from the induction of xanthine dehydrogenase (*AtXDH1*), the enzyme oxidising xanthine to uric acid, whereas mutation of this gene (*xdh1*) leads to plants which are more susceptible to dark treatment due to lack of allantoin/allantoate accumulation (Brychkova et al., 2008).

Other studies have shown that T-DNA insertions in allantoinase cause the constitutive accumulation of allantoin resulting in plant resistance to different abiotic stresses such as drought, salinity (NaCl) (Irani and Todd, 2016) and heavy metal cadmium toxicity (Nourimand and Todd, 2016). Elevated concentration of allantoin in *aln* mutants restricts the stress-induced ROS accumulation and consequently mitigates the negative effects of stress on plant growth (Brychkova et al., 2008; Watanabe et al., 2010). It has been indicated that allantoin applies its protective effect via inducing

Abbreviations: AAH, allantoate amidohydrolase; AAS, atomic absorption spectroscopy; ABA, abscisic acid; ALN, allantoinase; ALNS, allantoin synthase; ANOVA, analysis of variance; BG1, β-glucosidase 1; CAT, catalase; DAB, 3,3′-diaminobenzidine tetrahydrochloride; JA, jasmonic acid; MS, Murashsige and Skoog; NBT, nitroblue tetrazolium; NCED3, 9-cis-epoxycarotenoid dioxygenase; qRT-PCR, quantitative reverse transcription PCR; ROS, reactive oxygen species; SOD, superoxide dismutase; UO, uricase; XDH, xanthine dehydrogenase.

* Corresponding author. Department of Biology, 112 Science Place, University of Saskatchewan, Saskatoon, Saskatchewan, S7N 5E2, Canada.
E-mail address: chris.todd@usask.ca (C.D. Todd).

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stress signalling pathways such as abscisic acid (ABA) and jasmonic acid (JA). Elevated concentrations of allantoin in *aln* mutants stimulates the transcription of *NCED3* and post-transcriptional regulation of *BG1*, two key enzymes involved in ABA metabolism, leading to enhanced ABA accumulation in these plants (Watanabe et al., 2014). Moreover, *aln* mutants also exhibit an increased level of JA, activating JA-responsive genes. Accumulation of ABA and JA in allantoin-accumulating mutants induces a signal transduction cascade that alters the expression pattern of stress-responsive genes that regulate plant function under stress (Takagi et al., 2016; Watanabe et al., 2014).

In a previous study, we presented data showing reduced ROS levels in *aln-3* leaves in response to Cd treatment. This is due to activation of antioxidant enzymes, including superoxide dismutase (SOD) and ascorbate peroxidase (APX) (Nourimand and Todd, 2016). In the present study, we examine the impact of Cd on the growth, allantoin concentration, ureide metabolic gene regulation, ROS content, and antioxidant enzyme activity in allantoinase-negative and wild-type roots to see if allantoin plays a similar role in this tissue. Considering that roots are the first organ to come in contact with a wide range of soil contaminants, including Cd, we reason that evaluating whether roots and leaves have similar responses to Cd would also improve our understanding of the link between Cd tolerance and the role allantoin plays in plant abiotic stress response.

2. Material and methods

2.1. Plant materials, growth conditions and cadmium treatment

Col-0 and *aln-3* seeds were surface sterilized using 10% (v/v) bleach (sodium hypochlorite) for 10 min followed by 10 washes with sterile dH_2O. *aln-3* seeds were directly descended from the BC_2F_3 generation described by Irani and Todd (2016). Sterilized seeds germinated and grew on 0.5X Murashige and Skoog (MS) Basal Salt Mixture (PhytoTechnology Laboratories, Shawnee Mission, KS, USA) containing 1% (w/v) sucrose, 0.8% (w/v) agar, pH 5.7 adjusted with KOH. Plated seeds were incubated in the dark for 48 h at 4 °C and then transferred to a growth chamber with an 8 h/16 h day/night photoperiod, at 22 °C, 65% relative humidity and 70 μmol photons $m^{-2}\cdot s^{-1}$. Ten day-old seedlings were transplanted to 5 cm × 5 cm pots filled with Sunshine Mix 1 (SunGro Horticulture, Agawam, MA, USA). Pots were irrigated twice a week with tap water and fertilized using Hoagland solution (Hoagland and Arnon, 1950) once a week. Four-week-old plants were irrigated twice a week with 0, 500, 1000 and 1500 μM $CdCl_2$ dissolved in water, for three weeks. Root tissue was separated from shoot, washed and all soil particles removed before being frozen with liquid nitrogen and stored at −80 °C.

2.2. Quantification of allantoin

Allantoin content of Col-0 and *aln-3* roots were quantified using an Agilent Technologies 1200-series HPLC system equipped with a Biorad Aminex HPX-87H Ion Exchange Column, (300 mm × 7.8 mm) as described in Nourimand and Todd (2016).

2.3. RNA extraction, reverse transcription-PCR and q-PCR

Total RNA of Col-0 and *aln-3* roots were extracted using E.Z.N.A. Plant RNA Kit (Omega Bio-Tek, Norcross, GA, USA) following the manufacturer's instruction. 200 ng of RNA was applied to make cDNA using the QuantiTect Reverse Transcriptase Kit (Qiagen, Toronto, ON, Canada) according to the manufacturer's instructions. Reverse transcription PCR (RT-PCR) was performed using primers and conditions described by Nourimand and Todd (2016). Primers and PCR program employed in RT-qPCR were as described by Brychkova et al. (2008). Primer efficiencies for *ACT2*, *UO*, and *ALN* all fell between 95% and 100%.

2.4. Antioxidant enzyme and ROS assays

Activity of three antioxidant enzymes (superoxide dismutase, catalase and ascorbate peroxidase) were tested following the protocol described by Elavarthi and Martin (2010). Concentration of H_2O_2 and O_2^- were detected using 3,3′-diaminobenzidine tetrahydrochloride (DAB) and nitroblue tetrazolium (NBT) staining techniques following the protocol explained by Ramel et al. (2009).

2.5. Measurement of Cd in plant root tissue (AAS assay)

Oven-dried root tissue was used to ascertain the concentration of internal Cd. Sample preparation and atomic absorption assay were performed as described in Nourimand and Todd (2016) and Kwong et al. (2011).

2.6. Statistical analysis

All data shown are the mean of at least three independent replicates ± SEM. One-factor and two-factor ANOVA of Microsoft Excel were performed to determine the differences among four Cd concentrations, two genotypes and possible interactions between these factors. When significant differences were detected, a Tukey-Kramer post-hoc test as well as student's t-test were applied using the SPSS statistical program v.22.0 (www.ibm.com) and Microsoft Excel. Differences at $P \leq 0.05$ were considered significant.

3. Results

3.1. aln-3 roots grow more than Col-0 roots under Cd treatment

aln-3 mutants and Col-0 plants were irrigated with 500, 1000 and 1500 μM $CdCl_2$ dissolved in water and roots were cleaned and measured for fresh and dry weight after 21 days. Increasing Cd concentration not only decreased shoot growth and leaf expansion but also limited root development (Fig. 1A). Both fresh and dry weight of roots in *aln-3* mutants and Col-0 plants were negatively influenced by Cd, becoming more severe with higher Cd concentrations (Fig. 1B and C). However, the weight of *aln-3* roots were consistently greater than those in Col-0 plants under the same Cd level and two-way analysis of variance (ANOVA) indicated that root biomass is influenced by a significant interaction between both genotype and Cd treatment, indicating that the two genotypes responded differently to the three Cd concentrations.

3.2. Down-regulation of ALN and UO genes impairs allantoin accumulation in Cd-treated Col-0 roots

Allantoin content of mutant and wild-type roots was quantified using High Performance Liquid Chromatography (HPLC). Allantoin content of Col-0 roots remained unchanged at 500 μM $CdCl_2$ and decreased at 1000 and 1500 μM $CdCl_2$ (Fig. 2). In *aln-3* roots, allantoin content increased nearly 2-fold at 1000 μM $CdCl_2$ but was similar to untreated roots at 1500 μM $CdCl_2$. Uric acid content was lower than allantoin and varied with treatments in both genotypes, but no significant difference was observed (Supplemental Fig. S2).

To investigate the molecular mechanism underlying allantoin content of root in response to Cd stress, reverse transcription-PCR