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Research article

There is a direct link between allantoin concentration and cadmium tolerance in Arabidopsis

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ABSTRACT

Allantoin, an important intermediate of ureide metabolism, has been the subject of investigation recently due to its dual function in nitrogen recycling and abiotic stress response in plants. Allantoin appears to be the dominant ureide accumulating in response to different abiotic stresses, and mutants containing elevated allantoin concentrations exhibit a stress-tolerant phenotype due to limited reactive oxygen species (ROS) generation. Here we describe the involvement of allantoin in stress response and attempt to explain the regulatory mechanism(s) underlying allantoin function in plants. Growth of wild type Col-0 seedlings in the presence of exogenous allantoin improved root elongation in response to Cd treatment. Allantoin treatment of Col-0 seeds increases superoxide dismutase activity causing an enhanced seed germination and seedling growth following Cd exposure. Additionally, allantoinase-overexpressed (*ALNox*) lines, with lower levels of allantoin, exhibited more susceptibility to Cd treatment than Col-0 Arabidopsis, implying that there is a positive correlation between allantoin concentration and Cd resistance in plants. Growing ABA-insensitive (*abi*) mutants on allantoin-containing media and comparison between *abi* mutants and their wild-type backgrounds demonstrated that the potential regulatory function of allantoin does not require ABA at germination but may be ABA-dependent at later stages of seedling growth, suggesting a potential crosstalk between allantoin-mediated stress response and ABA signalling pathway in plants.

1. Introduction

Ureides derive from oxidative degradation of purines. Once cleavage of purine rings leads to xanthine generation, its metabolism in the cytosol, peroxisomes and endoplasmic reticulum (ER) generates these compounds with high nitrogen content, called ureides. Ureide catabolism releases nitrogen in the form of ammonia (NH_4^+), which is used to support plant growth and development (Zrenner et al., 2006; Werner and Witte, 2011). Allantoin ($C_4H_6N_4O_3$) is an important intermediate of the ureide pathway. It is formed in peroxisomes via the function of two enzymes: uricase, oxidizing uric acid to 5-hydroxyisourate, and allantoin synthase, which converts 5-hydroxyisourate to allantoin (Stasolla et al., 2003; Lamberto et al., 2010; Pessoa et al., 2010). Allantoin is then translocated to the ER where the next enzyme of this pathway, allantoinase, converts it to allantoate (Raso et al., 2007). These compounds are important in recovering purine nitrogen during senescence, but more recently allantoin has also been implicated in the plant cell's response to abiotic stress.

Evaluation of 15 Chinese rice (*Oryza sativa*) cultivars demonstrated that higher amounts of allantoin in rice is positively related to their more tolerance in response to low temperature and drought conditions (Wang et al., 2007). Likewise, in common bean (*Phaseolus vulgaris*) water limitation increases allantoate content of treated plants which is associated with the induction and suppression of two enzymes catalyzing allantoate production and degradation, respectively (Alamillo et al., 2010). Additionally, high light treatment (750 μmol photon m^{-2} s^{-1}) causes an increase in the ureide content of *Eutrema salsugineum* (*Thellungiella salsuginea*) when compared with a normal light exposure (250 μmol photon m^{-2} s^{-1}) (Malik et al., 2016).

Allantoin accumulation has been reported in Arabidopsis in response to different abiotic stresses including prolonged darkness (Brychkova et al., 2008), drought and mannitol-induced osmotic stress (Watanabe et al., 2014; Irani and Todd, 2016), NaCl (Irani and Todd, 2016; Lescano et al., 2016), high irradiance (Irani et al., 2017), and cadmium (Cd) (Nourimand and Todd, 2016). Suppression of xanthine dehydrogenase (*XDH*), which leads to decreased allantoin production

Abbreviations: ABA, abscisic acid; ALN, allantoinase; ALNS, allantoin synthase; ANOVA, analysis of variance; CAT, catalase; JA, jasmonic acid; MS, Murashsige and Skoog; RT-qPCR, reverse transcription quantitative PCR; ROS, reactive oxygen species; SOD, superoxide dismutase; UO, uricase; XDH, xanthine dehydrogenase

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causes increased reactive oxygen species (ROS) accumulation, chlorophyll degradation, early senescence and increased cell death in response to stress (Brychkova et al., 2008; Watanabe et al., 2010). Conversely, loss of function allantoinase knock-out lines (*aln* mutants) demonstrate constitutive allantoin accumulation and these plants exhibit improved stress tolerance, manifesting as decreased ROS production, decreased cell death, and improved plant growth in response to various abiotic stresses (Irani and Todd, 2016; Lescano et al., 2016; Irani et al., 2017). Elevated allantoin appears to mitigate the effect of these stresses through decreasing ROS accumulation. In response to exogenous cadmium, allantoinase-negative *aln-3* mutants exhibit increased antioxidant enzymes in both shoots and roots that help the plant to minimize ROS accumulation, conferring stress tolerance to these mutants in response to $CdCl_2$ (Nourimand and Todd, 2016, 2017).

Cadmium is an abiotic stressor that induces ROS generation and oxidative stress in plants (Polle and Schützendübel, 2003). Other metals, such as Zn and Pb, also cause allantoin accumulation in both roots and shoots of *Echium vulgare* (Dresler et al., 2017b), which also vary in allantoin content based on the heavy metal content of the soil where the plants were collected (Dresler et al., 2017a). Our previous experiments using Cd as a source of abiotic stress have focused on the role of allantoin in allantoinase-negative Arabidopsis plants that have constitutively higher internal allantoin levels (Nourimand and Todd, 2016, 2017). Exogenous allantoin has been shown to improve plant performance in response to other abiotic stresses, including elevated NaCl (Irani and Todd, 2018). To determine if application of allantoin is able to similarly protect Arabidopsis from Cd-induced oxidative stress we undertook this study to evaluate the relationship between allantoin content and Cd response/tolerance in wild type Arabidopsis. Additionally, allantoin-derived ABA accumulation reported by Watanabe et al. (2014) led us to question whether there is a potential interaction between the function of allantoin and ABA-mediated stress responses. To this end, ABA-insensitive (*abi*) mutants were used to investigate whether exogenous allantoin requires an intact ABA signalling pathway to improve plant performance, with a goal of moving closer towards identifying the mechanism(s) responsible for allantoin's protective function in plants.

2. Material and methods

2.1. Plant materials

Except as indicated below, *Arabidopsis thaliana* ecotype Col-0 was used as the wild type line for experiments. *ALNox* lines were generated by transforming Arabidopsis with *CaMV 35S::ALN* construct. Double digestion of pCAMBIA 1303 was carried out using NcoI and BstEII restriction enzymes. Ligation of *ALN* coding sequence (*ALN-CDS*) into pCAMBIA 1303 was performed using T4 DNA ligase (NEB, Ipswich, MA, US). A ligation mixture containing pCAMBIA 1303 + *ALN-CDS* was uses to transform *Escherichia coli* (DH5α) and Kanamycin (Kan)-resistant bacterial colonies were selected. Plasmid minipreps of pCAMBIA 1303 + *ALN-CDS* were done using the E.Z.N.A. Plasmid Mini Kit (Omega Bio-tek., Norcross, GA, US) according to manufacturer's protocols. After sequence confirmation obtained from Eurofin Genomics, *Agrobacterium tumefaciens* (GV1301) was transformed with pCAMBIA 1303 + *ALN-CDS*. Positive *A. tumefaciens* colonies were used to transform *Arabidopsis thaliana* (ecotype Col-0) following the floral-dip method (Zhang et al., 2006). Transformed seeds were identified by growing on MS plates containing 25 μg ml^{-1} Hygromycin (Hyg). Number of Hyg-resistance and Hyg-sensitive seedlings and their Chi-Square (X^2) analysis was used to test for the presence of a single transgene insertion (Rana and Singhal, 2015) and T3 seeds with a > 0.95 probability of a single transgenic event were selected as a true transformed line (*ALNox*) for further experiments.

Three homozygous ABA-insensitive (*abi*) mutants were also purchased from Arabidopsis Biological Resource Center (ABRC): *abi1-1* (CS22), *abi3-4* (CS6130) and *abi4-1* (CS8104). *A. thaliana* ecotypes Landsberg erecta (Ler-0) was used as control for *abi1-1* and *abi3-4* and Columbia (Col-0) for *abi4-1*, as their corresponding wild-type backgrounds.

2.2. Growth condition

0.5× Murashige and Skoog basal salt mixture (MS) (PhytoTechnology Laboratories, Shawnee Mission, KS, USA) containing 1% (w/v) sucrose, 1.2% (w/v) agar and KOH-mediated pH 5.7 was used as growth media. Allantoin (Sigma-Aldrich, Oakville, ON, Canada) with final concentration of 10 mM were added to autoclaved MS media to prepare MS + Aln and MS + Aln + Cd plates. Cadmium was also added to MS culture as $CdCl_2$ to achieve the interested concentrations for MS + Cd and MS + Aln + Cd plates. Sterilized seeds were transferred to MS plates and allowed to grow in a growth chamber with an 8 h light/16 h dark photoperiod, 70 μmol photons m^{-2} s^{-1}, constant 22° C and 65% relative humidity for two weeks. A short-day photoperiod was used as a standard experimental condition to maintain consistency with our previous studies (Nourimand and Todd, 2016, 2017).

Seed pre-treatments were performed by soaking Col-0 seeds in 10 mM allantoin solution (Aln-treated) or an equal volume of dH_2O as a control treatment (H_2O-treated) for 48 h and then transferred to growth media and grown as described above.

2.3. Seed germination and root length measurements, allantoin quantification

Radical emergence from the seed coat was considered as an indicator of seed germination in all experiments. Root length was measured using ImageJ software (version 1.46r) after photographing the two-week old seedlings. Average root length of 20 seedlings per petri dish was considered as a single replicate. High Performance Liquid Chromatography (HPLC) was used to quantify allantoin content of seedlings as described by Nourimand and Todd (2016).

2.4. Enzyme assay

Protein extraction and allantoinase activity assay was performed via colorimetric assays following the protocol explained by Duran and Todd (2012) and Nourimand and Todd (2016). Sample preparation and antioxidant enzyme assay were carried out as described in Elavarthi and Martin (2010) and Nourimand and Todd (2016).

2.5. Quantitative PCR (RT-qPCR) assay

Total RNA was extracted using the E.Z.N.A. Plant RNA Kit (Omega Bio-Tek) and cDNA was generated using QuantiTect Reverse Transcriptase Kit (Qiagen) according to the manufactures' protocols. This cDNA was used as DNA template for PCR analyses. Primers used in this study were described by Irani and Todd (2016) and qPCR was carried out using iCycler iQ5 System (Bio-Rad) while Evagreen dye (Biotium) served as DNA-binding dye. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) was used to calculate the relative gene expression. In all RT-qPCR assays *ACTIN2* (At3g18780) was used as an internal reference gene.

2.6. Statistical analysis

All data presented are the mean of at least three independent replications ± the standard error of the mean SEM. Significance between pairs of means were also analysed by Student's t-test (Microsoft Excel). One-factor and two-factor Anova were used to determine the significant differences between groups (treatments or genotypes) and were calculated using Microsoft Excel. Where significant differences were

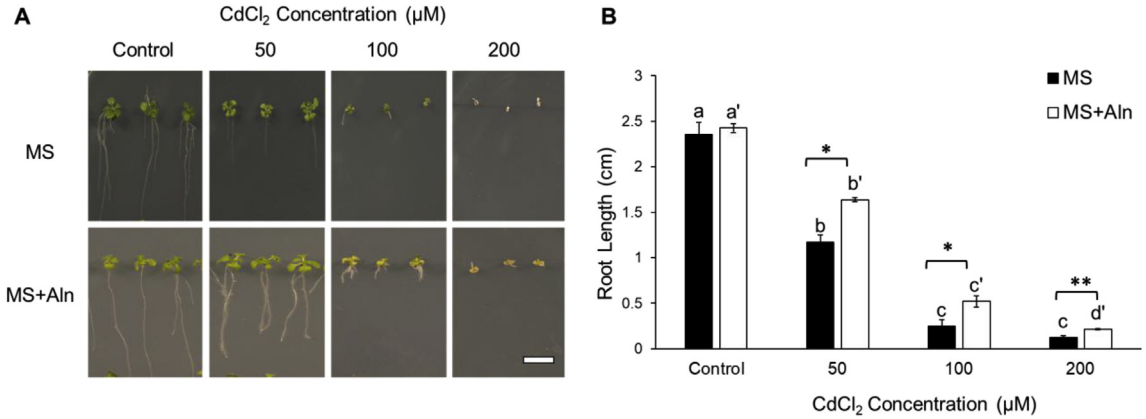

Fig. 1. Effect of exogenous allantoin (10 mM) on (A) seedling growth and (B) root elongation of Col-0 Arabidopsis in response to Cd treatment (50, 100 and 200 μM $CdCl_2$). Picture is representative of three independent experiments. Scale bar = 1 cm. Data are the mean of three independent replications ± SEM. Different letters are used to indicate significant differences ($P \leq 0.05$) between Cd concentrations on MS or MS + Aln plates, respectively. Differences between media types at the same concentration are indicated with an asterisk ($^*P \leq 0.05$ and $^{**}P \leq 0.01$). Aln, allantoin.

identified, a Tukey-Kramer post-hoc test was employed to identify significant differences among the samples (SPSS statistical program v.22.0. www.ibm.com). In all analyses $P \leq 0.05$ was reported as a significant difference.

3. Results

3.1. A positive effect of allantoin on wild-type root elongation following Cd treatment

Growing wild type Col-0 Arabidopsis seeds on MS media containing 10 mM allantoin (MS + Aln) and 50, 100, and 200 μM Cd improved plant growth and root elongation when compared with those grown in the absence of allantoin (MS) (Fig. 1A). The presence of allantoin alone in the growth media did not influence root length (Fig. 1B). However, once plants are exposed to Cd a significant difference was observed between MS- and MS + Aln-treated samples. Despite a Cd-induced decrease in the root length of both groups, seedlings grown in the presence of allantoin had longer roots than MS-grown seedlings. Increases were also seen in shoot biomass on MS + Aln, but only at 100 and 200 μM Cd (Figure S1).

3.2. A protective role for allantoin at early stages of seedling growth

The previous results led us to ask whether allantoin is effective at earlier stages of seedling development. To answer this, Col-0 Arabidopsis seeds were allowed to germinate on MS media and transferred to MS + Aln plates containing 50 and 100 μM $CdCl_2$ at different ages, two to five days after seed germination. MS plates containing the same Cd content were also used as controls. Once seedlings were two weeks old their root length were measured and compared between MS- and MS + Aln-treated samples. Consistent with previous observations, Cd decreased root growth in both groups (MS and MS + Aln). A difference between MS- and MS + Aln-exposed root length was only statistically significant when seedlings were transferred at an early stage of seedling development, two days after seed germination (Fig. 2), and only at 100 μM $CdCl_2$. No significant difference was observed between MS- and MS + Aln-grown seedlings for the seedlings transferred three to five days after seed germination (Supplementary Figs. S2A, B and C).

3.3. Effect of allantoin treatment of wild-type seeds on their germination and seedling growth

The varying response of seedlings depending on the timing of allantoin exposure prompted us to propose the hypothesis that exogenous allantoin may improve not only seedling growth, but also seed germination if it is employed before seeds germinate. To this end, Col-0 seeds were pre-treated with 10 mM allantoin or water controls, prior to plating on MS and MS + Aln plates with and without cadmium. As shown in Fig. 3, plants treated with $CdCl_2$, (MS + Cd and MS + Aln + Cd), had shorter roots than those of MS- and MS + Aln-treated samples four days after plating. In agreement with previous results, the presence of allantoin in the growth media (MS + Aln + Cd plates) alleviated the inhibitory effect of Cd (MS + Cd plates) on root elongation (Fig. 3B). However, at these two conditions there was a significant difference between allantoin- and H_2O-treated seedlings, with seeds which were soaked in allantoin exhibiting longer roots. The number of germinated seeds were also counted on days 4, 8 and 12 after transferring to the plates. The treatments had a noticeable effect on the seed germination on day four (Fig. 3C), but the percentage of germinated seeds reached the same level on days 8 and 12 for all treatments (data not shown). Among these growing conditions only 100 μM $CdCl_2$ (MS + Cd treatment) caused a significant decrease in germination of H_2O-treated seeds, while no meaningful change was observed in allantoin-treated seed at the same treatment (Fig. 3C). Additionally, allantoin-treated samples exhibited constantly higher seed germination in response to MS + Aln, MS + Cd and MS + Aln + Cd conditions when

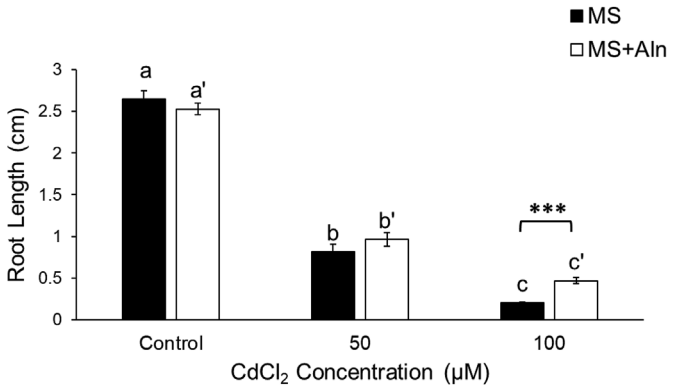

Fig. 2. Effect of exogenous allantoin (10 mM) and Cd (50 and 100 μM $CdCl_2$) on the root length of two-week old Col-0 Arabidopsis transferred on day two after seed germination. Data are the mean of three independent replications ± SEM. Different letters are used to indicate significant differences ($P \leq 0.05$) between Cd concentrations on MS or MS + Aln plates, respectively. Differences between media types at the same concentration are indicated with an asterisk ($^{***}P \leq 0.001$). Aln, allantoin.

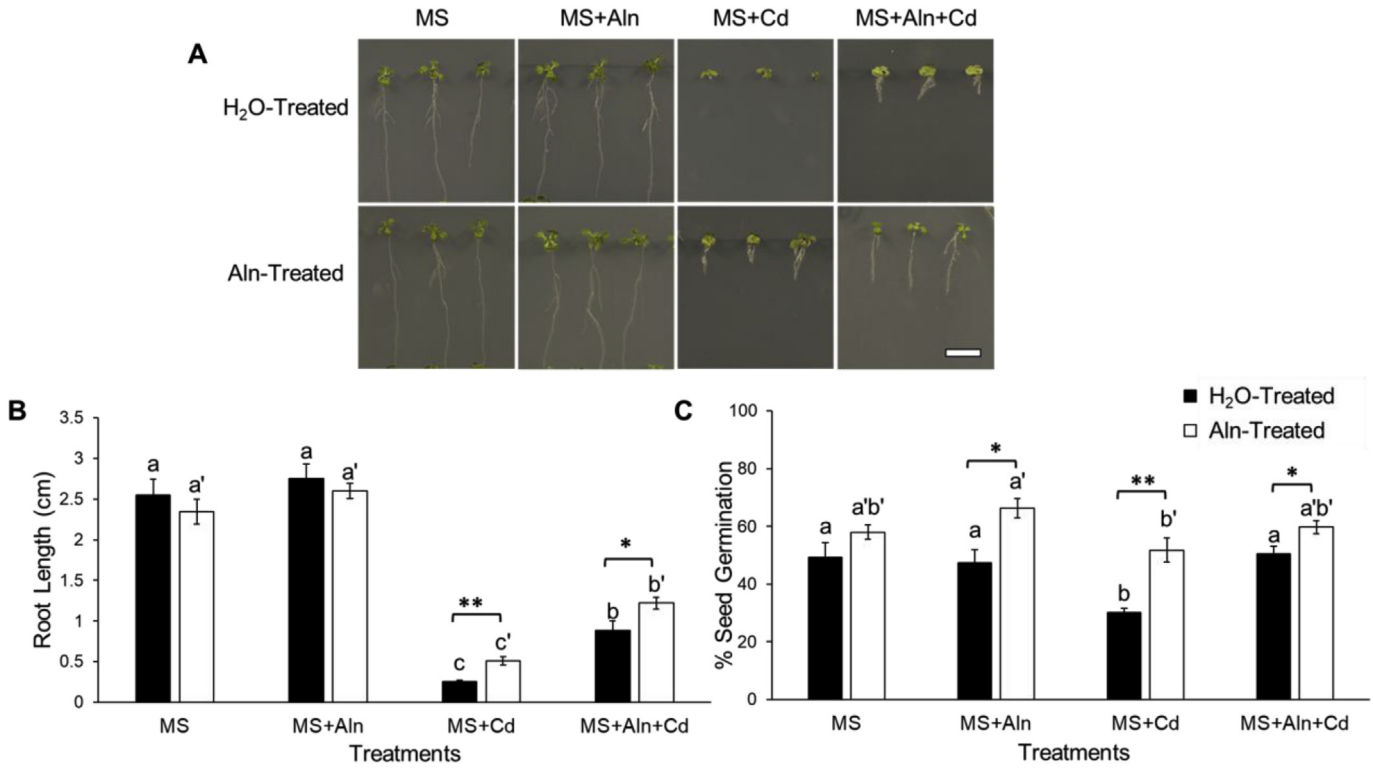

Fig. 3. (A) Plant growth of 100 µM $CdCl_2$-exposed Col-0 Arabidopsis after allantoin treatment (10 mM) of seeds. Picture is representative of three independent experiments. Scale bar = 1 cm. (B) Effect of allantoin treatment (10 mM) of Col-0 seeds on their root elongation in response to Cd (100 µM $CdCl_2$). (C) Germination of Col-0 Arabidopsis seeds four days following allantoin treatment (10 mM) and in response to Cd (100 µM $CdCl_2$). Data are the mean of three independent replications ± SEM. Different letters are used to indicate significant differences ($P \leq 0.05$) between media types for H_2O-treated and Aln-treated seedlings, respectively. Differences between H_2O and allantoin treatment on the same media are indicated with an asterisk ($^*P \leq 0.05$ and $^{**}P \leq 0.01$). Aln, allantoin.

compared with H_2O-treated seeds.

3.4. Allantoin-induced antioxidant activity in Col-0 seeds

Improved seed germination and seedling growth of Col-0 Arabidopsis following external administration of allantoin together with more active antioxidant enzymes in *aln-3* Arabidopsis which was previously reported (Nourimand and Todd, 2016, 2017), raising the question whether exogenous allantoin has the same stimulatory effect on the activity of antioxidant enzymes in Col-0 Arabidopsis seeds. To answer this question the activity of three antioxidant enzymes, superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT), were measured in allantoin-treated Col-0 seeds after 48 h soaking in 10 mM allantoin solution or dH_2O as a control condition. As presented in Fig. 4, three tested antioxidant enzymes had higher activity in response to allantoin treatment, while the difference between these two treatments, H_2O and allantoin, is only meaningful for SOD activity.

3.5. Characterization of ALN-overexpressed (ALNox) lines in response to Cd

Our previous experiments on *aln-3* mutants (Nourimand and Todd, 2016, 2017) along with the effect of exogenous allantoin on Col-0 Arabidopsis in present study, reinforced this idea that increased allantoin concentration (both *in vivo* and in vitro) causes Cd-tolerance in plants. However, it was still not clear whether there is a link between allantoin content of plants and their tolerance to Cd toxicity. To clarify this, allantoinase-overexpressed (*ALNox*) lines were generated. Transcript levels of *ALN* in *ANLox* lines is higher than that of Col-0 samples (Fig. S3A) and allantoinase enzyme activity was increased compared with Col-0 (Fig. S3B). Contrary to Col-0 seedlings, no detectable amount of allantoin was observed in *ALNox* lines (Fig. S3C). These data

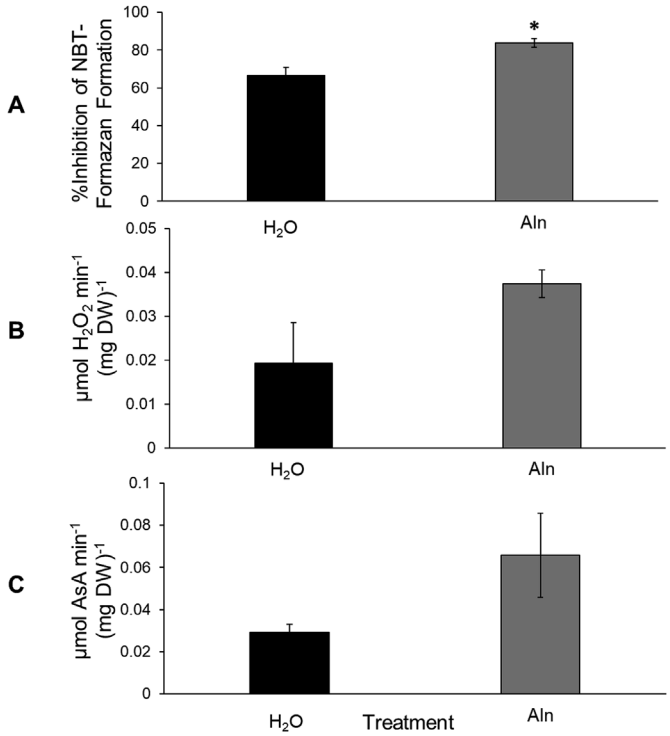

Fig. 4. Enzyme activity of (A) superoxide dismutase (SOD), (B) catalase (CAT) and (C) ascorbate peroxidase (APX) in Col-0 Arabidopsis seeds following allantoin treatment (10 mM). Data are the mean of three independent replications ± SEM. Significant differences between treatments are indicated with an asterisk ($^*P \leq 0.05$).

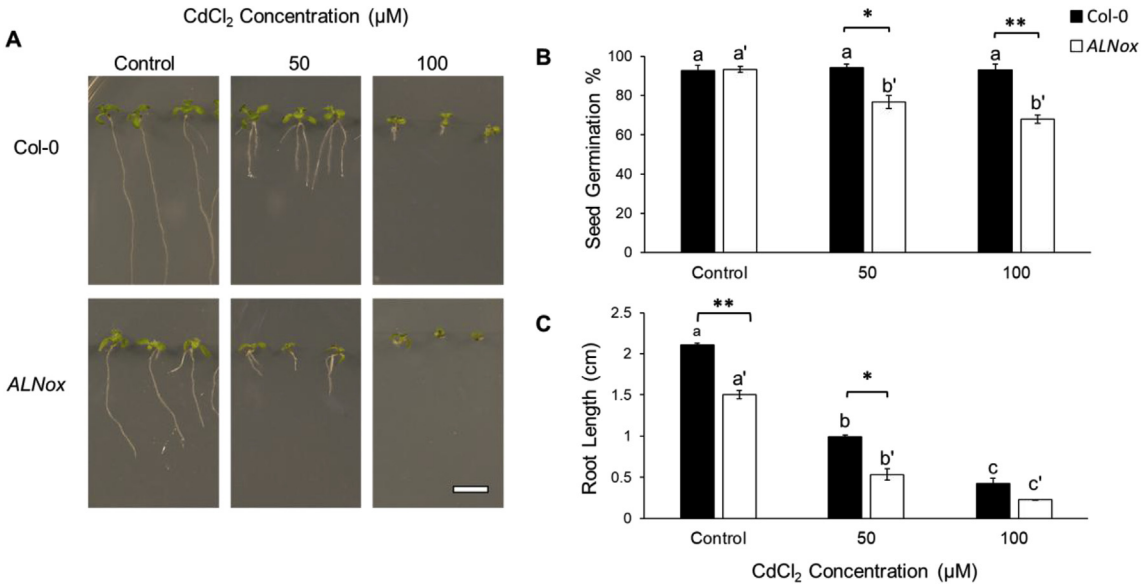

Fig. 5. (A) Plant growth, (B) seed germination, and (C) root length of Col-0 Arabidopsis and *ALNox* lines in response to Cd treatment (50 and 100 μM $CdCl_2$). Picture is representative of three independent experiments. Scale bar = 1 cm. Root length measurement and seed germination evaluation was carried out on day 14 of treatment. Data are the mean of three independent replications ± SEM. Different letters are used to indicate significant differences ($P \leq 0.05$) between Cd concentrations for Col-0 and *ALNox* seedlings, respectively. Differences between genotypes at the same Cd concentration are indicated with an asterisk (*$P \leq 0.05$ and **$P \leq 0.01$).

indicate that in *ALNox* lines the *ALN* gene expression increase leads to increased enzyme activity and suppressed allantoin accumulation. Therefore, we predicted that *ALNox* Arabidopsis should exhibit the opposite phenotype in comparison with *aln-3* mutants, namely sensitivity to abiotic stress.

In order to evaluate whether *ALN* overexpression alters the stress response in Cd-treated samples, *ALNox* and Col-0 Arabidopsis were exposed to 50 and 100 μM $CdCl_2$ and their seed germination and root length were measured. *ALNox* lines demonstrated impaired plant growth in comparison with Col-0 samples (Fig. 5A). 50 and 100 μM $CdCl_2$ significantly deacreased seed germination in *ALNox* lines, persisting to day 14 after plating, while these two Cd concentrations did not have a noticeable effect on the germination of Col-0 seeds (Fig. 5B). Root length measurement demonstrated that Cd inhibited root elongation in both genotypes, whereas *ALNox* lines exhibited considerably shorter roots than Col-0 seedlings throughout the experiment (Fig. 5C). There was no difference in shoot biomass between *ALNox* and Col-0 with Cd treatment, though *ALNox* shoots were smaller than control on 50 μM $CdCl_2$, whereas Col-0 seedlings did not show a significant decline until the concentration was 100 μM (Figure S4). HPLC analysis indicated that 100 μM $CdCl_2$ increased allantoin content of Col-0 seedlings, whereas no allantoin was observed in *ALNox* lines in response to 100 μM $CdCl_2$ treatment (Fig. 6A). Mean enzyme activity of *ALNox* plants was significantly greater than Col-0 in the absence of $CdCl_2$, but not when plated in the presence of 100 μM $CdCl_2$ (Fig. 6B).

3.6. Effect of external allantoin on Cd-exposed ALNox lines

Since allantoin improved Col-0 growth in the presence of Cd, we wished to determine whether allantoin can increase plant growth in Cd-treated *ALNox* Arabidopsis. Growing *ALNox* seeds on MS and MS + Aln plates demonstrated that although Cd has an inhibitory effect on root growth of both groups, there was not a significant effect between MS- and MS + Aln-exposed *ALNox* seedlings (Fig. 7A). Quantification of allantoin showed that once seedlings were grown on allantoin-containing plates (MS + Aln and MS + Aln + Cd), both Col-0 and *ALNox* Arabidopsis exhibited a considerable amount of allantoin with no meaningful difference between *ALNox* and Col-0 samples at this condition (Fig. 7B), likely representing extracellular allantoin, including that being transported in the vasculature. External allantoin also stimulated allantoinase activity in both genotypes with significantly higher value in *ALNox* lines at MS + Aln + Cd treatment (Fig. 7C).

3.7. Seed germination and root elongation of abi mutants in response to allantoin and Cd exposure

Allantoin-induced ABA accumulation in *aln* mutants and in response to exogenous allantoin (Watanabe et al., 2014) raised this question whether protective role of allantoin in response to abiotic stresses is mediated through an ABA-dependent or -independent mechanism. Therefore, ABA-insensitive (*abi*) mutants were employed to study the possible cross-talk between regulatory function of allantoin and ABA signalling pathway. Following experiment were designed based on the primary hypothesis that if the stress tolerance afforded by allantoin relies on ABA signalling pathway, external application of allantoin will not confer Cd resistance in ABA-insensitive (*abi*) mutants. Three *abi* mutants (*abi1*, *abi3* and *abi4*) (Koornneef et al., 1984; Ooms et al., 1993; Finkelstein, 1994; Bies-Etheve et al., 1999) and their wild-type backgrounds (Col-0 and Ler-0) were evaluated for their response to allantoin and cadmium, alone and in combination.

100 μM $CdCl_2$ treatment (MS + Cd) caused a slight decline in the seed germination of *abi* mutants and wild-type samples compared to MS alone (Fig. 8). However, this Cd-derived decrease is only significant for *abi 1*. In all samples (mutants and wild-types) MS + Aln + Cd treatment enhanced seed germination when compared with MS + Cd data. Root length of all seedlings decreased significantly in response to Cd treatment (MS + Cd) (Fig. 9). In Col-0 and Ler-0 MS + Aln + Cd improved root elongation in comparison with MS + Cd treatment, while this allantoin-induced root growth was not observed in *abi* mutants.

4. Discussion

The effect of Cd treatments on ureide metabolism and the protective function of allantoin in *aln-3* mutants has been demonstrated but leaves

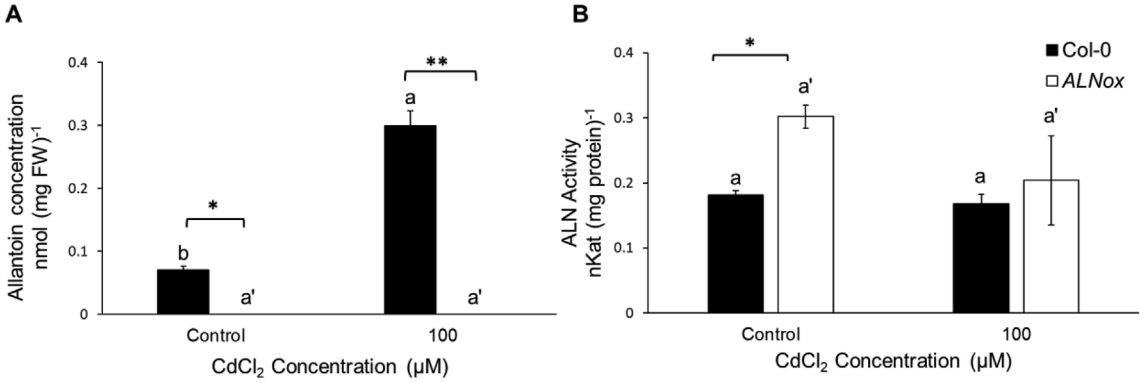

Fig. 6. Effect of 100 μM $CdCl_2$ on (A) allantoin content, and (B) Allantoinase activity of Col-0 and *ALNox* Arabidopsis. Data are the mean of three independent replications ± SEM. Different letters are used to indicate significant differences ($P \leq 0.05$) between Cd concentrations for Col-0 and *ALNox* seedlings, respectively. Differences between genotypes at the same Cd concentration are indicated with an asterisk (*$P \leq 0.05$ and **$P \leq 0.01$).

several questions regarding potential mechanisms. Our goal with this work was to investigate the potential positive effect of external allantoin on wild-type Arabidopsis, clarify the link between allantoin content and stress tolerance, and attempt to explain the association of ABA signalling pathway with the function of allantoin. Our results provide additional evidence for the protective effect of allantoin on early seedling growth and demonstrate the positive link between allantoin concentration and stress tolerance in plants.

4.1. Exogenous allantoin improve seed germination and seedling growth in Cd-treated wild-type Arabidopsis

Allelopathic properties have been suggested for allantoin in different studies, illustrating that allantoin exuded from corn cockle (*Agrostemma githago*) has a stimulatory effect on biomass production and yield of wheat (*Triticum aestivum*) (Gajic, 1966; Mallik and Williams, 2005; Wang et al., 2007). Likewise, allantoin released from *Memora peregrina* enhances seed germination in *Lactuca saliva* (Grassi et al., 2005; Wang et al., 2007). Not only plants, but also the microbial population of rhizosphere, such as actinomycetes, are influenced by the allantoin exuded from rice roots (Wang et al., 2007). A positive effect of external allantoin has been reported in different studies indicating that application of allantoin and allantoate minimize dark-induced ROS accumulation and chlorophyll degradation in wild-type Arabidopsis (Brychkova et al., 2008). Similarly, exogenous allantoin improves seed germination and seedling survival of rice grains at chilling and dehydration conditions (Wang et al., 2012). Although no antioxidant activity was reported for allantoin, lower ROS content and enhanced plant growth in *aln* mutants and in response to exogenous allantoin at stress conditions suggest that the function of this ureide is likely coupled with improved antioxidant system in plants.

Although different studies have been carried out on the positive effects of allantoin on plant stress tolerance, it is still ambiguous whether the stimulatory effect of allantoin on plant growth under stress conditions is related to its participation in metabolic processes or is associated with a regulatory or signalling function. In this study, 10 mM allantoin was chosen as a treatment. Lower concentrations (0.1 mM and 1.0 mM) of exogenous allantoin have been shown not to induce

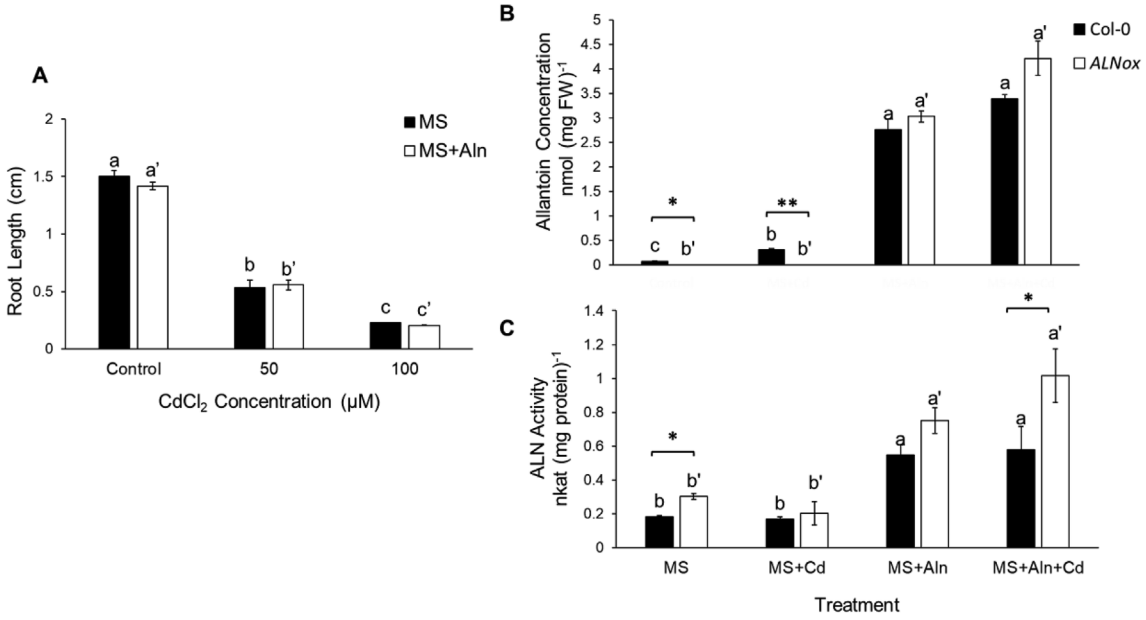

Fig. 7. (A) Root length of *ALNox* Arabidopsis in response to exogenous allantoin (10 mM) and different Cd concentrations (50 and 100 μM $CdCl_2$). Comparison between (B) allantoin content, and (C) Allantoinase activity of Col-0 and *ALNox* Arabidopsis following allantoin (10 mM) and Cd (100 μM $CdCl_2$) treatments. Data are the mean of three independent replications ± SEM. Different letters are used to indicate significant differences ($P \leq 0.05$) between Cd concentrations for MS and MS + Aln media, respectively (A), or to indicate significant differences between treatments for Col-0 and *ALNox* seedlings, respectively (B and C). Differences between genotypes on at the same media are indicated with an asterisk (B and C) (*$P \leq 0.05$ and **$P \leq 0.01$). ALN, allantoinase; Aln, allantoin.

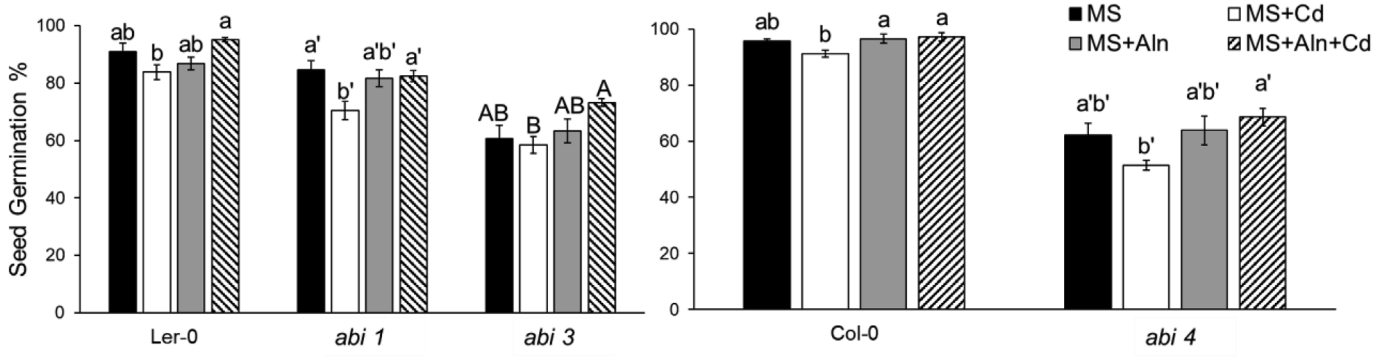

Fig. 8. Seed germination of *abi* mutants and wild-type Arabidopsis (Col-0 and Ler-0) in response to exogenous allantoin (10 mM) and Cd treatment (100 μM $CdCl_2$). Data are the mean of three independent replications ± SEM. Different letters show significant differences between treatments within each genotype ($P \leq 0.05$). Aln, allantoin.

allantoin accumulation to similar levels seen in *aln* mutants, even though they did have an effect similar to proline in protecting seedlings from 100 mM NaCl (Irani and Todd, 2018). Using 10 mM allantoin we attempted to mimic the effect of the loss of the allantoinase enzyme in *aln* mutants in our previous experiments with cadmium stress.

Since allantoin has four nitrogen atoms, in our experiment we have added the equivalent of an additional 40 mM N in the media. However, allantoin is not a particularly good source of nitrogen to Arabidopsis seedlings in culture. When fed 10 mM allantoin as a sole N source, Col-0 Arabidopsis seedlings do not develop normally on MS media, showing significant proliferation of root tissue, indicative of a nutrient stress (Todd and Polacco, 2006). Likewise, providing 5 mM allantoin as a sole nitrogen source in the growth culture, Desimone et al. (2002) reported that Arabidopsis seeds were able to germinate and complete their life cycle. However, these plants showed smaller leaves and symptoms of N-deficiency (higher root:shoot ratio) when compared with plants grown in the presence of equimolar N in the form of ammonium nitrate (10 mM) (Desimone et al., 2002). The ureide transporter gene, ureide permease (*UPS*), which has a high affinity for allantoin, is induced not only in the presence of allantoin, but also as a result of nitrogen shortage and stress conditions (Desimone et al., 2002; Lescano et al., 2016). Therefore, we suggest that though allantoin may function as an alternative nitrogen source in the absence of primary sources of N in the media, its role as a N fertilizer is likely minor, provided adequate accessible forms of N. Still, we cannot rule out the possibility that additional N contributed positively to the growth of seedlings when combined with the NH_4NO_3 and KNO_3 in the MS media.

In this study the effect of allantoin on Cd^{2+} uptake was not addressed. No specific transporter has been identified for Cd, and Cd is suggested to co-opt other cation transporters (such as Mg^{2+}, Ca^{2+} and Fe^{2+}) due to its similarity to these divalent elements (DalCorso et al., 2008; Gonçalves et al., 2009). It is reasonable to assume that Cd and allantoin uptake routes do not overlap, and we are unaware of any reports suggesting allantoin in the soil influences Cd uptake. However, quantifying Cd uptake by plants as well as employing a range of external allantoin concentrations could address this possibility in the future.

Consistent with our previous experiments, allantoin-induced Cd tolerance is mainly attributed to its stimulatory effect on antioxidant enzymes. Higher activity of SOD and APX in *aln-3* leaves (Nourimand and Todd, 2016) together with more active SOD and CAT enzymes in *aln-3* roots (Nourimand and Todd, 2017) result in Cd-tolerance in these mutants when compared with wild-type seedlings. Likewise, in the present study enhanced SOD activity in Col-0 seeds was observed following allantoin treatment implying that allantoin likely prepares the future seedling to confront stress and overcome Cd toxicity effectively via restricting superoxide accumulation and minimizing oxidative damage. Furthermore, the protective effect of allantoin at early stages of seedling growth (two days after seed germination) suggest that this ureide may also have a developmental stage-specific function in germinating seeds that is separate from its role in seedling growth. Considering that seed germination (Fig. 8) and root growth (Fig. 9) responded differently in the *abi* mutants, this may point to different mechanisms or signalling pathways for these two physiological functions, supporting a model which includes ABA-dependent and –independent functions (See section 4.4, below).

4.2. Overexpression of ALN induces Cd-sensitivity in ALNox lines

ALNox lines were constructed to evaluate the relationship between allantoin content of plants and their stress tolerance. Our results showed that overexpression of *ALN* imposes a Cd-sensitive phenotype

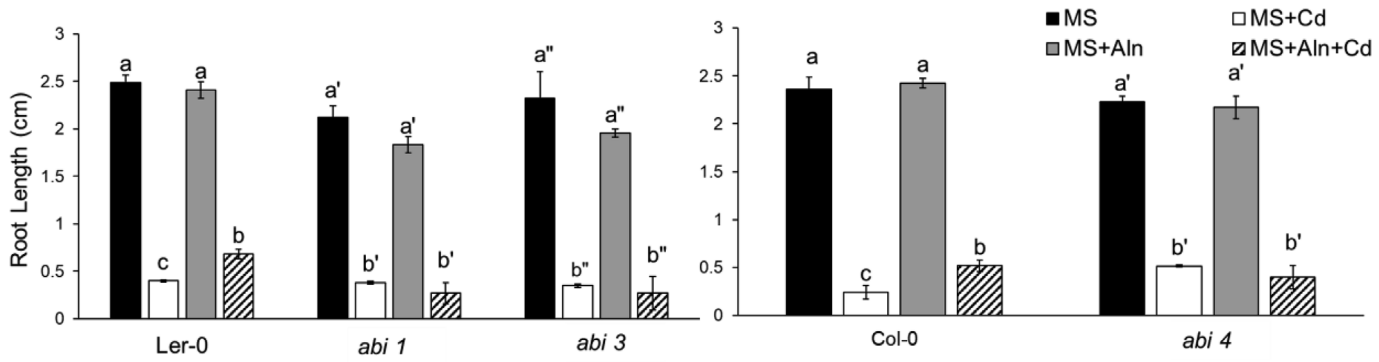

Fig. 9. Root length of *abi* mutants and wild-type Arabidopsis (Col-0 and Ler-0) in response to exogenous allantoin (10 mM) and Cd treatment (100 μM $CdCl_2$). Data are the mean of three independent replications ± SEM. Different letters show significant differences between treatments within each genotype ($P \leq 0.05$). Aln, allantoin.

on *ALNox* lines. Comparing *aln-3* mutants (Nourimand and Todd, 2016, 2017) and *ALNox* (this study) illustrates that these two genotypes function in an opposite manner, as might be predicted. Loss of functional allantoinase in *aln-3* mutants, results in allantoin accumulation and resistance to exogenous Cd (Nourimand and Todd, 2016) as well as other abiotic stresses (Irani and Todd, 2016; Irani et al., 2017), whereas overexpression of *ALN* causes decreased allantoin content and increased sensitivity to Cd (Fig. 5). Considering that *aln-3* and *ALNox* Arabidopsis exhibit reversed responses to stresses, it is reasonable to conclude that there is a positive correlation between allantoin concentration and stress tolerance in plants. Increased concentration of allantoin in *aln-3* mutants causes Cd-tolerance, whereas lower level of allantoin accumulation in *ALNox* enforces Cd-susceptibility. These findings are also consistent with results obtained from exogenous application of allantoin, intimating that increased allantoin content protects plants from harmful effects of Cd toxicity. Impaired stress response of Arabidopsis is also reported following *ALN* overexpression by using the promoter of *RD29A* (dehydration responsive gene), a stress-inducible gene. Enhanced expression of *ALN* under the control of *RD29A* promoter (*RD29A::ALN*) causes an increase in *ALN* expression and decreased allantoin accumulation in response to NaCl treatment (Lescano et al., 2016). This decline in allantoin content leads to decreased plant growth and weight in *RD29A::ALN* transgenic plants in respect with wild-type and *aln* mutants, agreeing with our observations. In comparing these two studies it is important to highlight the difference in growth conditions, including the longer photoperiod used by Lescano et al. (2016). While we employed a shorter photoperiod for consistency with our previous study, we might expect a more sever phenotype in the *ALNox* lines, with a longer day length and greater total irradiance. It is also interesting that the increase in cell-free activity measured in *ALNox* lines was comparable to wild-type under some experimental conditions (Figs. 6B and 7C), even though overexpressed allantoinase is clearly able to metabolize all endogenous allantoin (Figs. 6A and 7B). This leads us to speculate that there may be some level of post-transcriptional or post-translational regulation of this enzyme, with the caveat that cell-free extracts may not accurately reflect activities *in vivo,* but at present we have no data to support this.

4.3. Exogenous allantoin does not rescue ALNox Arabidopsis from Cd toxicity

Interestingly, exogenous availability of allantoin did not improve plant growth in *ALNox* which is in contrast with our observation in Col-0 samples. The presence of allantoin in growth culture increased allantoin concentration in *ALNox* seedlings, however, why this accumulated allantoin does not participate in stress response and Cd tolerance is not quite clear. Considering the reports characterizing ureides metabolism and different cellular compartments involved in ureide pathway, we propose that what we observed in allantoin-treated *ALNox* lines is likely attributed to subcellular localization of allantoin at this condition. Under normal conditions and in wild-type Arabidopsis allantoin is generated in peroxisomes and transferred to the ER. The enzyme allantoinase resides in the ER and catalyses allantoin degradation to produce allantoate (Hanks et al., 1981; Werner and Witte, 2011). Although allantoin quantification of MS + Aln-treated *ALNox* lines demonstrate high amounts of allantoin in these seedlings, it does not reveal where in the cell allantoin accumulates upon allantoin treatment. Additionally, despite enhanced allantoinase activity in *ALNox* Arabidopsis our data do not point to the subcellular location of this enzyme in *ALNox* lines. However, inefficient function of allantoin in *ALNox* lines lead us to this conclusion that the majority of absorbed allantoin by *ALNox* Arabidopsis are likely accumulated in extracellular spaces and the amount of allantoin that enters the cell are extensively degraded by already induced allantoinase enzyme. Therefore, allantoin is not accessible to cells or does not accumulate in cells to modify stress response and afford stress tolerance. Further experiments using promoter:reporter constructs may be required to elucidate the effect of external allantoin on stress response in *ALNox* lines. These results also identify a question that has yet to be addressed in this area. Specifically, in which subcellular compartment does allantoin have a physiological effect? In *aln* mutants it is generally assumed that the ER is the initial site of allantoin accumulation, since this is where allantoinase resides. However, there are no data that have been presented to suggest allantoin only accumulates in the ER. Elevated allantoin in the ER could lead to unfavorable conditions for ER-uptake and subsequently allantoin accumulation in the cytosol and in the peroxisomes. The idea that allantoin may have a signalling effect in the cytosol is interesting, since this is likely the first subcellular compartment where exogenous allantoin accumulates in wild-type plants. In the absence of a method to simultaneously measure or image allantoin in these three compartments *in vivo*, it is important to note that specifically where allantoin is functioning is yet unknown.

4.4. Impaired ABA sensing causes differential responses to allantoin and Cd in abi mutants

Protective effect of allantoin on plant function at stress conditions suggested a regulatory function for this ureide compound, acting as a signalling molecule that modulates stress response in plants (Guskov et al., 2004; Watanabe et al., 2014). Accumulation of ABA and JA, two stress response hormones, in *aln* mutants and in response to external allantoin proposes a possible connection between allantoin and other regulatory mechanisms such as ABA and JA signalling pathways (Watanabe et al., 2014; Takagi et al., 2016). However, it is still unknown whether allantoin acts as an inducer of ABA/JA production or its protective function is mediated through ABA pathway to modulate stress response in plants. According to obtained results from *abi* and wild-type samples, stimulatory effect of allantoin on seed germination is independent of ABA sensing, since wild-type and *abi* mutants exhibited the same responses to MS + Aln + Cd treatment when compared with MS + Cd exposure. Conversely, in two wild-type genotypes (Col-0 and Ler-0) MS + Aln + Cd-treated seedlings have longer roots than MS + Cd-exposed samples while this raise in root length is not observed in *abi* mutants, implying that protective effect of allantoin on root elongation is mediated through ABA signalling pathway. Therefore, existing data lead us to conclude that association of allantoin with ABA signalling mechanism is case-dependent. Some allantoin-mediated functions require the components of ABA pathway while others are regulated through an ABA-independent pathway, with a possible cross-talk between these two processes. Therefore, complementary experiments using double mutants, such as *aln/aba*, *aln/abi* and *ALNox/abi*, are required to explain the link between allantoin and ABA and illustrate their possible collaboration at stress conditions.

Contributions

M.N. and C.D.T. conceived and designed the experiments. M.N. carried out the experiments and collected the data. C.D.T. and M.N. analysed the data and wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2018.11.016.

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