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Allantoin accumulation in response to increased growth irradiance in *Arabidopsis thaliana*

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Abstract

Excess radiation is one of frequent natural environmental stresses that plants have to cope with on a daily basis. Therefore, plants have evolved many short- and long-term mechanisms to acclimate to high irradiance and tolerate it. Ureides, generated from purine degradation, have been proposed as compounds involved in environmental stress responses, including altered irradiance. In the present study, high irradiance was used to investigate ureide content and gene expression in *Arabidopsis thaliana*. *Arabidopsis* plants shifted to high irradiance showed high content of a specific ureide compound, allantoin. The accumulation of allantoin was associated with increased expression of uricase, an enzyme involved in its production. When an *Arabidopsis* mutant (*aln-3*), which constitutively accumulates elevated amounts of allantoin, was exposed to high irradiance, mutant plants demonstrated enhanced tolerance to the stress conditions compared to the wild-type plants. Our results provide evidence that accumulation of the allantoin might contribute in plants response to increased growth irradiance.

Additional key words: abiotic stress, allantoinase, high irradiance, ureides, uricase.

Introduction

In plant cells, ureides are commonly considered as nitrogen storage and transport compounds which recover nitrogen from purine rings. Moreover, in ureide-exporting legumes, the nitrogenous compounds originating from nitrogen fixation are transported from nodules as the ureides allantoin and allantoate (Todd *et al*. 2006). Several studies in plants have indicated that the metabolites of ureide metabolism increase as a response to various abiotic stresses (Brychkova *et al*. 2008, Alamillo *et al*. 2010, Coleto *et al.* 2014, Malik *et al*. 2016). More specifically, metabolic analysis of a number of desiccation-tolerant plants (Oliver *et el*. 2011, Yobi *et al*. 2013) and profiling of stress-inducible metabolites in rice (Degenkolbe *et al*. 2013, Nam *et al*. 2015, Wang *et al*. 2016) indicated that a ureide compound, allantoin, is associated with abiotic stress response. This compound has been also found to contribute in *Arabidopsis* tolerance to a number of abiotic stresses (Wantanabe *et al.* 2014, Irani and Todd 2016, Lescano *et al*. 2016, Nourimand and Todd 2016). During purine catabolism, allantoin is formed from xanthine and is catabolized to allantoate in a series of enzymatic steps that occur in the cytosol (xanthine dehydrogenase) and peroxisome (urate oxidase and allantoin synthase) (Werner and Witte 2011) as illustrated in Fig. 1.

We recently showed that growth irradiance increases the total ureide content in *Eutrema salsugineum* (Malik *et al*. 2016). *Arabidopsis* is a close relative of *Eutrema*, with up to 90 % nucleotide identity and similarity in life cycle as well as many morphological characteristics (Bressan *et al*. 2001, Amtmann 2009). However, *Arabidopsis* is less stress-tolerant compared to *Eutrema*,

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Abbreviations: AAH - allantoate amidohydrolase; ABA - abscisic acid; *ACT7* - Actin7; ALN - allantoinase; Chl - chlorophyll; F_v/F_m - variable to maximum chlorophyll fluorescence ratio (maximal quantum yield of PS II photochemistry); HI - high irradiance; HPLC - high performance liquid chromatography; MI - moderate irradiance; PPDF - photosynthetic photon flux density; ROS - reactive oxygen species; RT-PCR - reverse transcriptase-polymerase chain reaction; UO - uricase; XDH - xanthine dehydrogenase.
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which has shown high tolerance to drought, salinity, and freezing (Wong *et al*. 2005, Amtmann 2009). We hypothesize that ureides also accumulate in *Arabidopsis* under high irradiance in response to increased photooxidation. The aim of the current study was to investigate the effect of irradiance, photoperiod, and temperature, to identify if specific ureide compounds accumulate in response to high irradiance, as well as to determine changes in expression of associated genes in *Arabidopsis*. We also took advantage of an allantoinase-negative T-DNA insertion mutant and examined responses of this mutant to increased irradiance in comparison with wild-type plants.

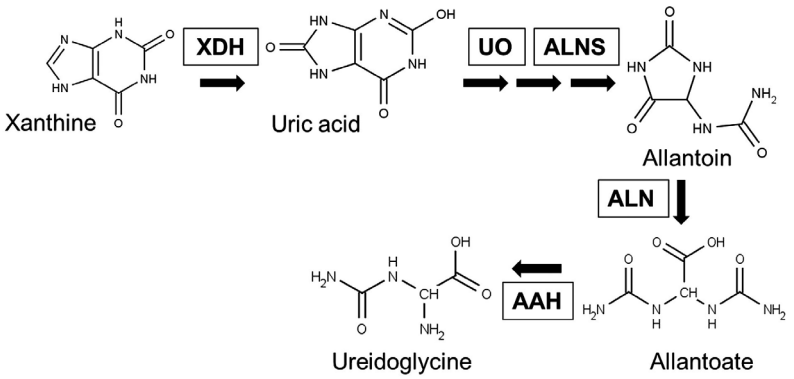

Fig. 1. Overview of allantoin metabolism from purines in *Arabidopsis*. Enzymes are shown in *boxes*. XDH - xanthine dehydrogenase, UO - uricase, ALNS - allantoin synthase, ALN - allantoinase, AAH - allantoate amidohydrolase. Enzymatic reactions take place in the cytosol (XDH), peroxisome (UO, ALNS), and endoplasmic reticulum (ALN, AAH), respectively.

Materials and methods

Plants and growth conditions: *Arabidopsis thaliana* L., ecotype Columbia (Col-0) was used for all experiments as the wild-type together with the previously characterized *aln*-3 *Arabidopsis* T-DNA insertion mutant (Irani and Todd 2016). Seeds were surface sterilized by soaking in commercial bleach containing 0.05 % (m/v) *Tween-20* for 8 min, followed by three rinses with sterile water. Seeds were sown on 0.8 % (m/v) *Bacto-agar* media plates (pH 5.7) containing half-strength Murashige and-Skoog (1962; ½ MS) basal salts (*PhytoTechnology Laboratories*, Shawnee Mission, KS, USA) supplemented with 1 % (m/v) sucrose. Plates were kept at 4 °C in the dark for 2 d and then placed in a growth chamber (*Conviron*, Winnipeg, MB, Canada) set at a 16-h photoperiod, an irradiance of 100 μmol (photons) m^{-2} s^{-1}, and a temperature of 22 °C. After 7 d, seedlings from the MS plates were transferred to 10-cm plastic pots containing *Sunshine Mix #1* (*Sun Gro Horticulture*, Vancouver, BC, Canada) and grown for additional 10 d. Then, two different growth irradiances moderate (MI, 250 μmol m^{-2} s^{-1}) and high irradiance (HI; 750 μmol m^{-2} s^{-1}) were tested for an additional 21 d at day/night temperatures of 22/10 °C and a 21-h photoperiod (Malik *et al*. 2016). All pots were irrigated with water as required and 200 mg dm^{-3} *20-20-20 Plant-Prod*® fertilizer (*Master Plant-Prod*, Ancaster, ON, Canada) was added to their water once a week.

High performance liquid chromatography (HPLC): Fully-expanded leaves (50 - 100 mg) were harvested. Detection of uric acid, allantoin, and allantoate was performed using an *Agilent 1200* HPLC system (*Agilent Technologies*, Mississauga, ON, Canada). The preparation of samples and HPLC procedures were done as described previously (Irani and Todd 2016).

Reverse transcriptase-polymerase chain reaction (RT-PCR): Total RNA was isolated from 25 mg of leaf tissue using an *E.Z.N.A* Plant RNA kit (*Omega Bio-Tek*, Norcross, GA, USA) following the manufacture's recommendations. Samples were eluted in sterile water and quantified using a *NanoDrop 2000* spectrophotometer (*Thermo Fisher Scientific*, Waltham, MA, USA). Gel electrophoresis using a 1 % (m/v) agarose gel run in 1× Tris acetate EDTA buffer (TAE) and stained with ethidium bromide was used to examine the quality of the RNA, which was used immediately or stored at -80 °C until use. Total RNA (1 μg) was used for first strand cDNA synthesis in a 20 mm^3 of reaction mixture using the *QuantiTect*® reverse transcription kit (*Qiagen*, Toronto, ON, Canada) following the manufacturer's protocols. Samples were analyzed using *TopTaq* DNA polymerase (*Qiagen*) using the primers described by Irani and Todd (2016). PCR conditions were as follows: initial denaturation at 95 °C for 3 min; 32 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 40 s, extension at 72 °C for 1 min; followed by a final extension at 72 °C for 7 min. All analyses were carried out using an *iCycler* thermocycler (*Bio-Rad Laboratories*, Hercules, USA). *ACTIN7* (At5g09810) was used as a reference gene. A *GeneRuler* 1 kb DNA ladder (*Thermo Fisher Scientific*) was used for sizing. PCR products were visualized on a 1.2 % (m/v) agarose gel run in 1× TAE and stained with

ethidium bromide following standard protocols (Sambrook and Russell 2001).

A real-time quantitative (q)PCR analysis of gene expression was done according to Brychkova *et al.* (2007) with modifications described by Irani and Todd (2016) using *ACTIN2* (At3g18780) as a reference. All primers sequences were those described by Brychkova *et al.* (2008). The mean expression on day 0 was normalized to 1 and was used as a basis to compare the expression of HI treated samples. Relative fold changes in expression of genes were calculated using the relative $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Chlorophyll (Chl) fluorescence and pigment content: The variable to maximum Chl *a* fluorescence ratio which characterizes maximal quantum yield of PS II photochemistry (F_v/F_m) was determined in Col-0 and *aln-3* leaves after 0, 7, 14, and 21 d of growth under MI or HI. Day 0 represents the day before starting the irradiance treatments. Measurements were determined *in vivo* using a *PAM-2000* portable chlorophyll fluorometer (*Heinz WalzGmbH*, Effeltrich, Germany) and F_v/F_m was calculated as $(F_m - F_0)/F_m$, where F_0 and F_m represent the minimal and maximal fluorescence at the dark-adapted state, respectively. Measurements were made on detached leaves at room temperature following a 15 min dark-adaptation.

At the same time, content of Chl *a+b* and anthocyanins was determined in Col-0 and *aln-3* leaves. The content of Chl *a+b* was determined in 80 % (v/v) acetone (HPLC grade; *Fisher Chemicals*, Pittsburgh, PA, USA) extracts prepared from rosette leaves using the equations of Lichtenthaler and Wellburn (1983).

Anthocyanin extraction was based on the method of Lange *et al*. (1970) with minor modifications according to Noh and Spalding (1998). The rosette leaf tissue was weighed and placed in microcentrifuge tubes containing extraction buffer consisting of 1-propanol (HPLC grade; *EMD Chemicals*, Etobicoke, ON, Canada), HCl (*Fisher Chemicals*), and water (18:1:81, v/v/v). Samples were ground using plastic pestles and placed in a boiling water bath for 3 min. The tubes were incubated in the dark at room temperature for 2 h and then centrifuged at 18 000 *g* for 2 min. Anthocyanins in the supernatant were quantified spectrophotometrically and calculated as difference in absorbances A_{535} - 2.2 A_{650} (Lange *et al*. 1970).

Statistical analysis: Data are presented as means ± standard error of the mean (SE). Mean values were compared using Student *t*-test or one-factor *ANOVA* by *SPSS v. 22.0* software (*IBM*, Markham, CA). When significant differences were detected, a Tukey HSD post-hoc test was applied. Differences at $P < 0.05$ were considered as significant.

Results

In order to determine effect of different growth irradiance on ureide content, wild-type 17-d-old *Arabidopsis* plants were grown under MI or HI for 21 d and ureide content of fully-developed rosette leaves was quantified using HPLC. No changes in uric acid or allantoate were detected in response to different irradiances. However, allantoin increased in the leaves already 4 d after transferring plants to the HI conditions. Allantoin content reached a maximum of 0.72 µmol g^{-1}(d.m.) on day 7, dropping on days 10 and 14, respectively, and after 21 d no allantoin was detected in the leaves (Fig. 2). However, the appearance of the leaves suggested that they were death after 21 d under HI. There were not any detectable amounts of allantoin or other ureides also in the leaf samples which were taken from plants grown under MI.

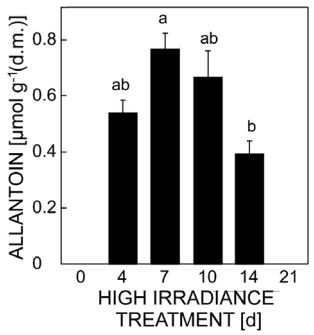

Fig. 2. Allantoin content in Col-0 leaves after 0, 4, 7, 10, 14, and 21 d of HI treatment detected by HPLC. Day 0 indicates the day before HI treatment. Means ± SEs, $n = 3$. Different letters denote significant differences in allantoin content (Tukey HSD test, $P < 0.05$).

Expressions of genes encoding uricase (*UO*), allantoinase (*ALN*), and allantoate amidohydrolase (*AAH*) were analyzed by real-time qPCR to determine whether their expression was influenced by different irradiance. In response to HI the relative expression of *UO* started to increase on day 4 and up to the day 10 (Fig. 3). The transcription of all of the genes was increased on day 10 but then reduced on day 14 (Fig. 3). Further, the expression of ureide metabolic genes was measured on day 10 by in plants grown under MI or HI and the samples taken from plants grown under HI generally showed higher ureide gene expressions (Fig. 1 Suppl.).

After approximately 7 d of HI, plants started to show phenotypic changes compared to those in MI. The main visible morphological changes under HI included hastened reproductive development, thickened leaves, reduced chlorophyll content, and enhanced content of anthocyanin which resulted in the purple leaf colour. On day 14 after HI treatment, it was clear that Col-0 plants lost more chlorophyll and accumulated more purple pigments compared to *aln-3* plants. On day 21 after HI

treatment the plants started reproductive development, further accumulated purple pigments, and begun to desiccate (Fig. 2 Suppl.). Under MI, the both plant lines

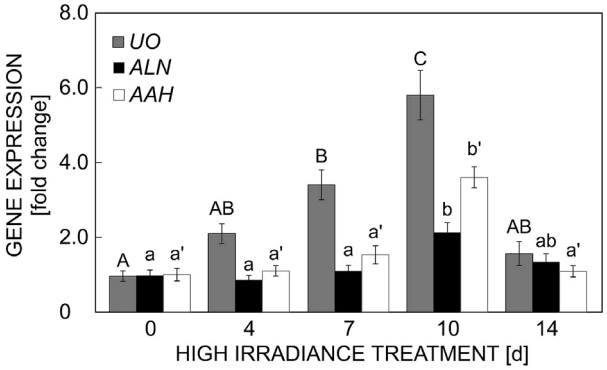

Fig. 3. Real-time qPCR analysis of genes encoding ureidase (*UO*), allantoinase (*ALN*), and allantoate amidohydrolase (*AAH*). Fold-change in expression of *UO*, *ALN* and *AAH* in leaf tissue of Col-0 plants on day 0, 4, 7, 10, and 14 after HI treatment. *ACTIN2* was used as internal control. Means ± SEs, $n = 3$. Different upper or lower case letters represent significant differences among days of high irradiance treatment for *UO* (A,B,C), *ALN* (a,b,c), and *AAH* (a',b',c') determined by Tukey HSD test ($P < 0.05$).

WT and *aln-3* developed more slowly, and were visually undistinguishable.

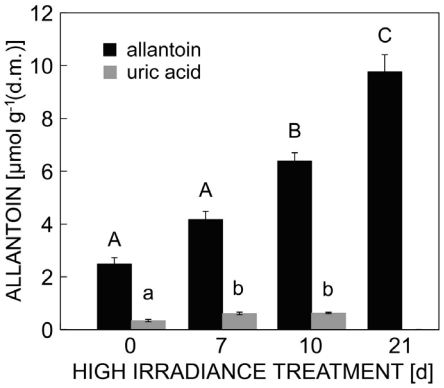

Fig. 4. Allantoin and uric acid content in *aln-3* leaves on day 0, 7, 10, and 21 after HI treatment. Day 0 represents the day before shifting the plants to the HI condition. Means ± SEs, $n = 3$. Different letters denote significant differences in allantoin (A,B,C) or uric acid (a,b) content among time points (Tukey HSD test, $P < 0.05$).

Considering the phenotypic difference between the Col-0 and *aln-3* under HI, the content of ureide compounds was also measured in *aln-3* mutant plants exposed to HI. Allantoin and uric acid were detected in *aln-3* leaves on day 0 and other time points after HI treatment (Fig. 4). No allantoate was detected in *aln-3*, consistent with previous reports (Irani and Todd 2016, Nourimand and Todd 2016). Uric acid was present at a low level throughout the experiment but was not detectable on day 21. Allantoin content in the mutant was increased incrementally to a maximum amount on day 21 (Fig. 4).

Under MI the content of Chl *a+b* and anthocyanins as well as F_v/F_m ratio did not differ significantly between Col-0 and *aln-3* plants at any time point when analyzed by Student *t*-test (Table 1). However, Chl *a+b* content and F_v/F_m ratio were significantly higher in *aln-3* on day 14 of the HI treatment compared to Col-0 (Table 1). Anthocyanin content was significantly higher in the Col-0 leaves compared to *aln-3* on day 14 and 21 of HI treatment (Table 1), agreeing with visual observations.

Discussion

Suitable irradiance is one of the essential factors contributing to plant growth and development. However, excess of radiation energy can be a cause of reactive oxygen species (ROS) production (Asada and Takahashi 1987, Wituszyńska and Karpiński 2013). The ROS can negatively affect macromolecules, such as lipids, nucleic acids, and proteins, and if the plant cannot prevent the photooxidative stress, eventually leading to senescence and cell death (Foyer and Noctor 2009, Gill and Tuteja 2010, Pintó-Marijuan and Munné-Bosch 2014). The photosystem II can be inactivated and damaged by excess of radiation energy, the phenomenon known as photo-inhibition (Aro *et al*. 1993).

An increase in total ureide content was reported in *Eutrema* by Malik *et al*. (2016) under high growth irradiance associated with long photoperiod. A 2.5-fold increase in total ureides was observed when Yukon ecotype of *Eutrema* was grown under HI, compared to plants grown under same photoperiod but MI. Here, the amounts of three ureide compounds, uric acid, allantoate, and allantoin were measured individually in plant leaves using HPLC. Under the same growth conditions as used Malik *et al.* (2016), *Arabidopsis* plants exposed to HI accumulated allantoin within a few days after the

treatment (Fig. 2). However, no detectable allantoin (or uric acid, or allantoate) was observed in plants grown under MI. Since the photoperiod and day/night temperature was similar under moderate and high irradiance, accumulation of allantoin in leaves in *Arabidopsis* under these conditions is primarily the effect of irradiance.

Table 1. The content of chlorophyll *a+b* [mg g^{-1}(f.m.)] and anthocyanins [ΔA g^{-1}(f.m.)] and the maximal quantum yield of photosystem II photochemistry (F_v/F_m) of Col-0 and *aln-3* leaves on day 0, 7, 14, and 21 after moderate (MI) and high irradiance (HI) treatments. Means ± SEs, $n = 3$. Significant differences between Col-0 and *aln-3* plants were determined by *t*-test (* - $P < 0.05$, ** - $P < 0.01$), nd - not detected.

Parameter	Time [d]	MI		HI	
		Col-0	*aln-3*	Col-0	*aln-3*
Chl *a+b*	0	0.70 ± 0.04	0.67 ± 0.04	nd	nd
	7	0.73 ± 0.04	0.73 ± 0.05	0.54 ± 0.03	0.51 ± 0.03
	14	0.85 ± 0.06	0.88 ± 0.07	0.33 ± 0.02	0.46 ± 0.02**
	21	0.64 ± 0.03	0.63 ± 0.03	0.06 ± 0.02	0.10 ± 0.01
Anthocyanins	0	nd	nd	nd	nd
	7	0.31 ± 0.02	0.28 ± 0.02	2.50 ± 0.3	2.30 ± 0.3
	14	0.53 ± 0.03	0.48 ± 0.05	35.00 ± 1.1	28.00 ± 0.1**
	21	0.78 ± 0.02	0.80 ± 0.03	65.00 ± 0.6	61.00 ± 0.7*
F_v/F_m	0	0.80 ± 0.00	0.80 ± 0.00	nd	nd
	7	0.81 ± 0.01	0.81 ± 0.00	0.76 ± 0.01	0.76 ± 0.00
	14	0.81 ± 0.00	0.81 ± 0.01	0.46 ± 0.01	0.57 ± 0.02*
	21	0.80 ± 0.01	0.80 ± 0.01	nd	nd

A number of studies have also reported that dark treatment of plants resulted in accumulation of allantoin and allantoate, *e.g.*, in *Phaseolus vulgaris* and *Funaria hygrometrica* (Engelbrecht 1955, and Hartmann and Arnold 1974, as cited by Castro *et al.* 2001) as well as in *Arabidopsis* (Brychkova *et al*. 2008). In response to different photoperiods, accumulation of allantoin increased in comfrey roots under longer photoperiod (Castro *et al*. 2001). Vitória and Mazzafera (1999) reported that in *Coffea arabica* and *C. dewevrei*, caffeine can be slowly catabolized to xanthine, and in turn, xanthine generates ureides. Therefore, higher content of allantoin and allantoate has been was found in these plants. In *C. arabica*, higher content of allantoin has been found under HI (Pompelli *et al*. 2013). The present research also shows that allantoin accumulated in the leaf tissue of *Arabidopsis,* which does not form caffeine. Thus, accumulation of allantoin under HI can be considered as a plant response to excess radiation, though at the later time points we cannot exclude the possibility that ureides accumulate in response to tissue damage or cell death.

Brychkova *et al*. (2008) reported that the transcription of uriede synthesis genes xanthine dehydrogenase1 (*XDH1*) and *UO* is enhanced in *Arabidopsis* under prolonged darkness whereas the transcription of ureide catabolism genes (*ALN* and *AAH*) is decreased. Malik *et al*. (2016) showed lower expression of enzymes involved in allantoate degradation in response to HI, indicating that growth irradiance might affect the ureide content in *Eutrema*. Here, the increase in the expression of *UO* on day 4, with no change in expression of *ALN,* is likely to lead to the accumulation of allantoin in *Arabidopsis* leaves under HI (Figs. 2, 3). These results are in parallel with our observation of higher transcription of *UO* in *Arabidopsis* under drought stress (Irani and Todd 2016) and excess of cadmium (Nourimand and Todd 2016). Interestingly, the HI differed from these reports in that both *ALN* and *AAH* were also induced on day 10. We take this to indicate that either these genes are induced by allantoin accumulation, or that the accelerated development of the plants under HI also causes developmentally-regulated resource recycling pathways, such as purine metabolism, to be turned on to redistribute N from nucleic acids in vegetative tissues to reproductive organs.

Watanabe *et al*. (2014) reported that two T-DNA insertion mutants, *aln-1* and *aln-2,* accumulate constitutive amounts of allantoin and show better seedling growth or survival rate in response to air-drying or osmotic stress. Likewise, *aln-3* show the enhanced tolerance to salinity and prolonged drought (Irani and Todd 2016). A higher F_v/F_m ratio was observed in *aln-3* plants compared to the wild-type plants under drought stress, whereas *aln-3* leaves accumulated less H_2O_2 and O_2. Here, the *aln-3* plants also exhibited better performance under HI on day 14, as shown by higher amounts of Chl *a+b* and F_v/F_m ratio (Table 1). The *aln-3* plants also accumulated less anthocyanin than the wild-type, 14 and 21 d after HI treatment (Table 1). One of the most common biochemical effects of HI is accumulation of anthocyanins, which protect leaves from photo-

oxidative damage (Gould *et al*. 2010) and/or help plants to acclimate to HI (Page *et al*. 2012). Less anthocyanin accumulation in *aln-3* may be related to lower sensitivity of the mutant to high irradiance. Thus, the Col-0 plants which are more susceptible to HI-induced damage than *aln-3* start to accumulate anthocyanins earlier and in greater amounts. In contrast, constitutively high content of allantoin in this mutant also led to the higher tolerance to HI.

Various studies demonstrated that the contribution of allantoin to stress protection might be related to mediation of ROS accumulation (Gus'kov *et al*. 2004, Brychkova *et al*. 2008) and/or mediation of ABA production (Watanabe *et al*. 2014). However, as a recent study of Takagi *et al*. (2016) shows a possible role of allantoin in activating the jasmonate signaling in the biotic stress responses. Malik *et al*. (2016) suggests that the increase amount of ureides under HI might help *Eutrema* to acclimate to irradiance and to enhance the tolerance to photoinhibition. Thus, ureide accumulation is proposed to be a plant defence mechanism (Malik *et al*. 2016). At present, the subcellular compartmentation and functions of allantoin are still not fully elucidated. Here we demonstrate that allantoin accumulated in *Arabidopsis* leaves under HI probably due to altered expression of genes encoding allantoin synthesis and degradation enzymes. Together with the better performance of *aln-3* under HI we suggest that allantoin is probably involved in a protective mechanism against oxidative stress induced by HI.

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