Growth irradiance affects ureide accumulation and tolerance to photoinhibition in *Eutrema salsugineum* (*Thellungiella salsuginea*)


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**Abstract**

Plants are able to acclimate to their growth light environments by utilizing a number of short- and long-term mechanisms. One strategy is to prevent accumulation of excess reactive oxygen species that can lead to photoinhibition of photosynthesis. Ureides, generated from purine degradation, have been proposed as antioxidants and involved in certain abiotic stress responses. *Eutrema salsugineum* (*Thellungiella salsuginea*) is an extremophilic plant known to exhibit a high degree of tolerance to a variety of abiotic stresses that invariably generate reactive oxygen species. In the present study we have investigated the possible role of the ureide metabolic pathway during acclimation to growth irradiance and its conference of tolerance to photoinhibition in *Eutrema*. Ureide accumulation was greater under high light growth which also conferred tolerance to photoinhibition at low temperature as measured by the maximal quantum yield of PSII photochemistry. This may represent an adaptive plastic response contributing to the extreme tolerance exhibited by this plant. Our results would provide evidence that ureide accumulation may be involved in abiotic stress as another defence mechanism in response to oxidative stress.

**Additional key words:** acclimation; allantoin; antioxidant; reactive oxygen species.

**Introduction**

Reactive oxygen species (ROS) are by-products of normal metabolism and include the hydroxyl radical (•OH), superoxide anion (O$_2^{−}$), hydrogen peroxide (H$_2$O$_2$) and singlet oxygen (O$_2$(1S)). While ROS have been shown to play an important signaling role, they are also highly reactive and if remained unchecked they can cause wide-spread damage to cellular components and structure. In order to prevent the oxidative damage, the plant controls ROS concentrations through a complex scavenging system consisting of diverse enzymatic and non-enzymatic mechanisms which include low-molecular mass antioxidants such as ascorbic acid (ascorbate) and glutathione (Asada and Takahashi 1987, Foyer and Noctor 2009, Gill and Tuteja 2010, Karuppanapandian et al. 2011, Suzuki et al. 2012).

Exposure to high light and absorption of energy in excess of what can be effectively consumed or dissipated by chloroplastic metabolism can lead to an inactivation of photosynthesis known as photoinhibition (Powles 1984, Aro et al. 1993). Variations in growth environments and the quantity of incident light occur continually, over time-scales ranging from seconds to months. Thus, plants have developed a wide array of both short- and long-term photoprotective and repair mechanisms/strategies at the whole plant, leaf or cellular/organelle levels to deal with excessive light (Demmig-Adams and Adams 1992). Photoinhibition is generally thought of as short-term light stress, growth under high light and the associated excess excitation energy presents more of a long-term challenge.
Plants are able to acclimate their photosynthetic apparatus by modulating their thylakoid architecture and protein distribution dynamically according to their growth light environments.

This usually involves changes in light-harvesting antenna size, reaction centre stoichiometry, contents of electron transport components and Calvin cycle enzymes. These responses have been well documented for many plant species (Anderson 1986, Anderson et al. 1995, Walters 2005).

*Eutrema salsugineum* (Pall.) Al-Shehbaz and Warwick (also known as *Thellungiella salsuginea* and *Thellungiella halophila*) is a native crucifer that is closely related to the genetic model plant *Arabidopsis thaliana* (Koch and German 2013). While both *Eutrema* and *Arabidopsis* are in the Brassicaceae, they differ greatly in their ability to tolerate adverse environmental conditions. *Eutrema* is often referred to as an extremophile, owed to a high capacity to withstand various abiotic stresses such as freezing, water deficit, and salinity in comparison to *Arabidopsis* (Inan et al. 2004, Griffith et al. 2007, Amtmann 2009, Guevara et al. 2012). Many ecotypes have evolved under diverse stress-prone environmental conditions and their stress tolerance behavior is thought to be appropriate to their natural habitats due, in part, to the high degree of adaptive plasticity shown by *Eutrema* (Amtmann 2009, Guevara et al. 2012, Kazachkova et al. 2013). The Yukon ecotype, originating from the saline meadows in the Yukon Territories, Canada, a subarctic and semiarid region, also thrives under conditions of very low soil nitrogen (< 1 mM; Guevara et al. 2012). While *Eutrema* has been used extensively for salinity studies, there has been relatively little work performed investigating photosynthetic responses and tolerance to high light stress or acclimation to growth irradiance (Wong et al. 2006, Stepieen and Johnson 2009, Sui and Han 2014).

Purine compounds perform essential metabolic roles and are integral components for many cellular processes (Stasolla et al. 2003, Zrenner et al. 2006). Ureides are nitrogen containing compounds generated from the purine degradation pathway. The first common intermediate of purine catabolism is xanthine which is oxidized to urate by xanthine dehydrogenase (XDH; EC 1.17.3.2, 1.17.1.4) for which two genes exist in *Arabidopsis* (Hesberg et al. 2004). Urate is further metabolized to the ureide allantoin, through the action of urate oxidase (UO; EC 1.7.3.3) and two subsequent enzymatically catalyzed conversions. Plants possess a set of enzymes which further break down allantoin, ultimately releasing CO₂ and ammonia (NH₃). The enzyme allantoinase (ALN; EC 3.5.2.5) converts allantoin to allantoate, followed by allantate amidohydrolase (AAH; EC 3.5.3.9) which converts to allantoate to ureidoglycine which is enzymatically degraded to ureidoglycylate and NH₃ by ureidoglycine aminohydrolase (UGlyAH; EC 3.5.3.26). In turn, ureidoglycylate is hydrolyzed to glyoxylate, CO₂ and two molecules of NH₃ by ureidoglycylate amidohydrolase (UAH; EC 3.5.1.116) (for review see Werner and Witte 2011). In symbiotic legumes, ureides are involved in the storage of fixed nitrogen and its translocation from N₂-fixing nodules to other tissues and organs (Schubert 1986, Todd et al. 2006). For a variety of non-N₂-fixing species, purine catabolism and ureide formation is also thought to contribute to the recycling/remobilization of nitrogen from source to sink organs (Reinbothe and Mothes 1962, Werner and Witte 2011). In addition, several studies in *Arabidopsis* using mutants in the purine degradation pathway have indicated that the metabolites of ureide metabolism (allantoin and allantoate) could act as potential antioxidants under abiotic stress conditions (Brychkova et al. 2008, Watanabe et al. 2010, 2014; Ventura et al. 2011). This is supported by recent metabolomic and phytochemical studies examining allantoin accumulation in response to water deficit and salinity that have also implicated a role for ureides in reducing ROS accumulation (Oliver et al. 2011, Ventura et al. 2011, Yobi et al. 2013). In contrast, another study looking at the function of allantoin in rice grains suggested that while allantoin accumulation did not serve directly as an antioxidant, it did show a positive correlation with seedling survival under low temperature and drought stress conditions (Wang et al. 2012).

The Yukon ecotype of *Eutrema* is also tolerant to nitrogen stress, a phenotype consistent with the prevailing conditions in its native habitat (Kant et al. 2008, Guevara et al. 2012). Kant et al. (2008) were able to show that *Eutrema* was highly efficient in the mobilization of resources in poor or degraded soils in comparison to *Arabidopsis*.

While not examined by Kant et al. (2008), ureides may be involved, consistent with their role in nitrogen recycling and mobilization. Given the potential role of ureides as antioxidants, we hypothesized that ureide accumulation could be playing a protective role in response to oxidative stress, contributing to the overall high degree of abiotic stress tolerance observed in *Eutrema*. Here, we described an investigation into the effects of growth irradiance on ureide accumulation in the Yukon ecotype of *Eutrema* and examined if this accumulation conferred any tolerance to high light (photoinhibitory) treatments at low temperature. Our results would provide evidence that ureides may be involved as an additional class of compound for protection against photo-oxidative stress.

**Materials and methods**

**Plant material and growth conditions:** Seeds of *Eutrema salsugineum* (Pall.) Al-Shehbaz and Warwick (Yukon ecotype, stock no. CS22664) were obtained from the Arabidopsis Biological Resource Centre (ABRC,
Columbus, OH, USA) and germinated in 6.5-cm plastic pots containing Sungrow Sunshine LG3 Mix germinating soil medium (Sun Gro Horticulture Inc., Vancouver, BC, Canada) which were placed in plastic tray flats. Plants were grown in controlled environment chambers (Conviron, Winnipeg, MB, Canada) using a day/night temperature (22/10°C) and a light/dark cycle (21/3 h) previously established for the Yukon etype of Eutrema (Griffith et al. 2007). Two different growth irradiances (determined at soil level) were used: moderate light (ML) and high light (HL) which corresponded to PPFDs of 250 and 750 μmol(photon) m–2 s–1, respectively. Aerial portions of single plants were harvested and dried for a minimum of 48 h at 90°C or until constant mass was obtained for the determination of dry mass (DM). Total leaf area (LA) measurements were performed using ImageJ (Schneider et al. 2012; http://imagej.nih.gov/ij/). Specific leaf mass (SLM) was calculated as mg(DM) cm–2. Plants were maintained and fertilized as described by Gray et al. (2003).

Ureide extraction and determination: Plant tissue (50–100 mg of fresh mass, FM) from fully expanded leaves was harvested and homogenized using a mortar and pestle in 10 volumes of ice-cold deionized H2O. The homogenate was centrifuged at 4°C and 14,000 rpm for 5 min to remove cell debris. The supernatant was removed and centrifuged for an additional 5 min at 4°C and 14,000 rpm. The resultant crude extract was kept on ice and used immediately to estimate a total ureide content.

A colorimetric assay of differential glyoxylate derivatives was performed to quantify total ureides as indicated in Table 1. ACT7 was used as a reference gene. A GeneRuler 1 kb DNA ladder (Thermo Scientific) was also used for sizing.

Photoinhibition and chlorophyll (Chl) fluorescence: The maximal quantum yield of PSII photochemistry (Fv/Fm) was determined in vivo using a hand-held portable fluorometer (FluoPen FP100; Photon Systems Instruments, Brno, Czech Republic) and calculated as (Fv – Fo)/Fm, where Fo and Fm represent the minimal and maximal fluorescence yields of the dark-adapted state, respectively. Measurements were made on detached leaves at room temperature following a 15 min dark-adaptation as described previously (Chytyk et al. 2011).

Photoinhibition of photosynthesis was induced in a cold room set at 2°C under ambient air conditions as described by Gray et al. (2003). Detached leaves were floated adaxial side up in a divided Petri dish containing deionized water. Susceptibility to photoinhibition was quantified by monitoring changes in Fv/Fm as a function of a 2 h exposure to PPFD of 1,750 μmol(photon) m–2 s–1 from 300 mg of leaf tissue using a urea-LiCl method (Kansal et al. 2008) with modifications. The samples were quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) followed by gel electrophoresis to examine the quality of the RNA. Agarose gels 1% (w/v) were run in 1× TAE buffer and stained with ethidium bromide according to standard procedures (Sambrook and Russell 2001). Samples were stored at –80°C and used as templates for subsequent sqRT-PCR.

Transcript levels were established by sqRT-PCR using an iCycler thermocycler (Bio-Rad Laboratories, Hercules, CA, USA) and a Verso 1-Step RT-PCR ReadyMix™ Kit (Thermo Fisher Scientific) as recommended by the supplier. Total RNA (60 ng) was amplified with gene-specific primer pairs (final concentration of 200 nM each forward and reverse) in 25 μl reactions. PCR products were visualized on a 1% (w/v) agarose gel run in 1× TAE and stained with ethidium bromide using standard procedures (Sambrook and Russell 2001). Primers were designed based on gene orthologs in Arabidopsis using the Primer-BLAST tool at the National Center for Biotechnology Information (NCBI; Ye et al. 2012) and are indicated in Table 1. ACT7 was used as a reference gene. A GeneRuler 1 kb DNA ladder (Thermo Scientific) was also used for sizing.

Table 1. Oligonucleotide primers used for sqRT-PCR. F – forward; R – reverse; AAH – allantoate amidohydrolase; UGlyAH – ureidoglycine aminohydrolase; ACT7 – actin7.

<table>
<thead>
<tr>
<th>Arabidopsis gene</th>
<th>Abbreviation</th>
<th>Sequence [5’→3’]</th>
<th>Size [bp]</th>
</tr>
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<tbody>
<tr>
<td>At4g20070</td>
<td>AAH</td>
<td>R: TTCAGGTTCGCCACACAAACCA</td>
<td>872</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F: AAGGGACGAAGCTTAGCACA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TAGCATTGGCGCAGTTGAG</td>
<td></td>
</tr>
<tr>
<td>At4g17050</td>
<td>UGlyAH</td>
<td>F: GCCCTTTACCTGATGGACAA</td>
<td>611</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: AGCTTTTCCGAGTGCAC</td>
<td></td>
</tr>
<tr>
<td>At5g09810</td>
<td>ACT7</td>
<td>F: GATATTCGACCGACTTGTTGAC</td>
<td>187</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CAGGTTCGATTGGATACTCCAGA</td>
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Results and discussion

Growth characteristics of Eutrema under ML and HL growth regimes: Since the irradiance conditions utilized resulted in different growth rates, plants were harvested at different times which corresponded to approximately 27 and 24 d after germination for ML and HL growth regimes, respectively. Growth kinetic analyses have indicated that despite minor differences in growth phenotype, plants of these chronological ages were at the same point in their growth cycle (Lobo and Gray, unpublished results). Overall, growth under HL resulted in plants with a 1.4-fold increase in SLM at these developmental ages compared to the ML conditions (Table 2). These results are consistent with leaf-level traits seen in plants acclimated to high and low irradiance for many species (Boardman 1977, Givnish 1988).

The changes occurring at the chloroplast level during acclimation to light have been examined intensively and are in part, reflected by changes in the ratio of Chl \( a/b \) (Anderson 1986, Walters 2005). Total Chl, estimated on a FM basis, demonstrated 37% decrease for plants grown at HL when compared to those developed under the ML growth regime which was accompanied by a 1.9-fold increase in the Chl \( a/b \) ratio, similar to what has been observed for acclimation to growth irradiance previously (Table 2; Boardman 1977, Anderson 1986). Anthocyanin accumulation in leaves is thought to protect against photoinhibitory damage caused by high irradiance either by light attenuation or antioxidant effects (Chalker-Scott 1999, Havaux and Kloppstech 2001, Neill and Gould 2003). In Eutrema, minimal changes were observed in bulk anthocyanins or total Car contents (xanthophylls and carotenes; Table 2). Car are involved in the prevention of photooxidation and the nonphotochemical dissipation of excess light energy through the xanthophyll cycle pigment interconversions (Demmig-Adams and Adams 1992). These results are not unexpected as we did not determine the epoxidation state of the xanthophylls which is more reflective of photoprotection than are Car pool sizes (Demmig-Adams and Adams 1992).

Ureide accumulation in Eutrema is dependent on growth irradiance: The data presented in Fig. 1A demonstrate an interesting trend with regard to ureide accumulation in leaf tissue developed under varying irradiance conditions. Growth at HL resulted in a 2.5-fold increase in total ureides when compared to the ML regime (Fig. 1A). Besides the Yukon ecotype of Eutrema, ureide accumulation was also observed in the ecotype Shandong grown at HL (data not shown). To our knowledge, this is the first report of ureide accumulation

Table 2. Leaf pigment contents for Eutrema (Yukon ecotype) developed at different growth irradiance. Plants were grown under either 250 (ML) or 750 \( \mu \text{mol} \text{ photon} \text{ m}^{-2} \text{ s}^{-1} \) (HL) PPFD. Values represent means ± SD (\( n = 3 \)). Different letters within columns indicate a significant difference at \( P=0.05 \) based on a Student’s \( t \)-test. Car – carotenoids; Chl – chlorophyll; DM – dry mass; FM – fresh mass; HL – high light; ML – moderate light; SLM – specific leaf mass.

<table>
<thead>
<tr>
<th>Growth regime</th>
<th>SLM [mg(DM) cm(^{-2})]</th>
<th>Total Chl [( \mu g \text{ g}^{-1}(\text{FM}) )]</th>
<th>Chl ( a/b )</th>
<th>Total Car [( \mu g \text{ g}^{-1}(\text{FM}) )]</th>
<th>Anthocyanin [( \mu g \text{ g}^{-1}(\text{FM}) )]</th>
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<tr>
<td>ML</td>
<td>2.94 ± 0.32(^{a})</td>
<td>1,255.79 ± 66.95(^{a})</td>
<td>3.56 ± 0.13(^{a})</td>
<td>204.53 ± 10.88(^{a})</td>
<td>9.60 ± 0.58(^{a})</td>
</tr>
<tr>
<td>HL</td>
<td>3.97 ± 0.29(^{b})</td>
<td>794.80 ± 100.010(^{b})</td>
<td>6.78 ± 1.18(^{b})</td>
<td>198.42 ± 8.18(^{a})</td>
<td>12.64 ± 2.67(^{b})</td>
</tr>
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</table>
in response to growth irradiance, although a previous study has implicated photoperiod as a factor in allantoin accumulation (Castro et al. 2001).

The expression level of two genes (AAH and UGlyAH), involved in allantote degradation, were analyzed by sqRT-PCR to determine whether the effect of growth irradiance could influence the concentration of ureides by the transcriptional regulation of these genes (Fig. 1B). Both genes, AAH and UGlyAH, showed a clear up-regulation in response to HL growth (Fig. 1B). In addition, ALN and XDH2 were absent under ML conditions but showed a very faint increase under the HL growth regime (data not shown). Genes involved in ureide synthesis and degradation have been shown to be induced upon stress conditions. Higher ureide concentrations, mainly in form of allantoin, were previously reported in shoots and leaves of common bean (Phaseolus vulgaris) during drought treatment and this was not a consequence of suppression of allantote degradation, but rather, an induction of biosynthesis regulated at the transcript level (Alamillo et al. 2010, Coleta et al. 2014). While we were unable to determine specifically which ureide was contributing to this increase, as only bulk measurements were performed, it also appears that in Eutrema growth irradiance may influence ureide accumulation at the level of transcription. Our experiment only indicated light responsiveness and did not allow us to speculate as to whether anabolic or catabolic processes are responsible for the observed accumulation in Eutrema.

Tolerance to photoinhibition: Exposure to high light and absorption of energy in excess of what can be effectively consumed or dissipated by chloroplast metabolism can lead to an inactivation of photosynthesis known as photoinhibition (Powles 1984, Aro et al. 1993).

Excessive ROS production can also lead to oxidative damage of PSII and a reduction in photosynthetic capacity (Aro et al. 1993). Furthermore, environmental stresses (salinity, cold, heat and drought) have all been shown to accelerate photoinhibition by limiting the photosynthetic fixation of CO2, resulting in ROS accumulation and decreased PSII repair (Takahashi and Murata 2008, Murata et al. 2012). Detached leaves of Eutrema were exposed to a PPFD of 1,750 μmol(phonon) m–2 s–1 at low temperature (2°C) to examine responses to photoinhibition. Similar values of Fv/Fm (ranging from 0.75 to 0.80) were obtained prior to the photoinhibitory treatment for plants grown under either the ML or HL regimes (Fig. 2). Although HL plants generally presented slightly lower values, no significant differences were observed. Following photoinhibition, Fv/Fm had decreased by approximately 50 and 14% for plants grown at ML and HL, respectively (Fig. 2). Thus, in Eutrema, acclimation to increased growth irradiance appeared to maintain optimal photochemical efficiency and increase the tolerance to photoinhibition as has been reported earlier for other species (Anderson and Osmond 1987, Anderson et al. 1995, Walters 2005).

Ureides and abiotic stress: The accumulation of allantoin and allantote are frequently in leguminous species as response to water limitation (Serraj et al. 1999, King and Purcell 2005). Watanabe et al. (2010) reported that in Arabidopsis, the silencing of key genes (XDH1 and XDH2) in the ureide pathway negatively influenced seedling biomass as well as cell survival rate when exposed to drought stress conditions. Moreover, mutant plants accumulated higher concentrations of H2O2 than wild type, suggesting that the intermediates of ureide metabolism might be involved in protection of the plant in drought by an antioxidative mechanism (Watanabe et al. 2010). A recent study by Watanabe et al. (2014) using a wide variety of mutants in the ureide degradation pathway studied the role of purine intermediates in response to drought and osmotic stress. Not only did allantoin content increase in wild type plants in response to stress conditions, but an ALN mutant, which constitutively accumulates allantoin, was more tolerant to the stresses than the wild type (Watanabe et al. 2014).

![Fig. 1. Effect of growth irradiance on ureide content and transcript level of ureide metabolic genes in leaves of Eutrema (Yukon ecotype). Plants were grown under either 250 (ML) or 750 μmol(photon) m–2 s–1 (HL) PPFD. A: Total ureide content. Total ureides are the sum of allantoin, allantote, ureidoglycolate and glyoxylate and are expressed in glyoxylate equivalents. Values represent means ± SE of three biological replicates. Bars with different letters are significantly different at P=0.05 based on a Student’s t-test. B: Transcript level of genes involved in allantote degradation. A representative image is shown. FM – fresh mass; HL – high light; ML – moderate light; AAH – allantote amidohydrolase; UGlyAH – ureidoglycine aminohydrolase; ACT7 – actin7.](image-url)
were grown under either 250 (ML) or 750 μmol(mol photon) m⁻² s⁻¹ (HL) PPFD. Responses were determined by monitoring changes in the Fv/Fm ratio prior photoinhibition (black bars) and post photoinhibition (white bars). Values represent means ± SD (n = 8). Bars with different letters are significantly different at P=0.05 based on a one-way ANOVA. Fv/Fm – maximal quantum yield of PSII photochemistry; HL – high light; ML – moderate light.

The phytohormone abscisic acid (ABA) is an important signaling hormone as plays a well-established role in mediating plant responses to abiotic stress (Nakashima et al. 2014). Watanabe et al. (2014) suggested that increased allantoin content elevated ABA concentrations, which in turn elicited ABA-mediated stress responses, thus linking allantoin accumulation and ABA-dependent signaling, also shown by Alamillo et al. (2010). Although we did not examine ABA in this study, it seems likely that contribution of allantoin in abiotic stress responses is both regulatory (ABA accumulation) and perhaps a more direct role in the detoxification of ROS.

Metabolic changes during abiotic and biotic stresses are associated with the remobilization of plant metabolites which is accelerated by catabolic activities (Sagi et al. 1998, Werner and Witte 2011). The use of a metabolic intermediate, such as a ureide, in stress responses has been suggested to facilitate rapid responses to environmental fluctuations whereby stress-induced inhibition or down-regulation allows the temporary accumulation of an intermediate with protective or regulatory properties and facilitates environmental acclimation and adaptation (Sagi et al. 1998, Watanabe et al. 2014). Ureides acting as ROS scavengers under stress would be consistent with their role in nitrogen recycling and mobilization, thereby supplying a nitrogen source under stress conditions (Werner and Witte 2011).

Conclusion: Our results with Eutrema demonstrated that ureides accumulated in leaf tissue during acclimation to high irradiance and this was correlated with increased tolerance to photoinhibition in these HL-grown plants. Our preliminary data indicated genes in the pathway are light responsive, suggesting regulation at the level of transcription. We suggest that ureides may be involved as another protective mechanism for ROS scavenging and/or ABA-dependent signaling in response to oxidative and possibly other abiotic stress responses.

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