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Short Communication

Arabidopsis *xanthine dehydrogenase* mutants defective in purine degradation show a compromised protective response to drought and oxidative stress

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Abstract Although generally recognized for its role in nitrogen recycling and remobilization, purine catabolism might also be involved in the plant response and adaptation to environmental stress. Previously, we demonstrated that allantoin, a major purine intermediary metabolite in stressed plants, stimulates abscisic acid production and activates stress-responsive gene expression, leading to increased tolerance to abiotic stress in Arabidopsis seedlings. Here, we show that dysfunction of purine degradation, as a result of knocking down the key enzyme xanthine dehydrogenase, severely reduced the survival of Arabidopsis under progressive drought conditions and significantly decreased the tolerance to superoxide-mediated oxidative stress. The enhanced stress sensitivity of the knockdown mutants likely resulted from defective stress responses, because the drought-induced accumulation of the cellular protectant proline was compromised in the knockdown plants, which also showed lower mRNA levels of *P5CS1*, the gene encoding the rate-limiting enzyme for proline biosynthesis. When exogenously applied to wild-type Arabidopsis, allantoin and its precursor urate were able to elicit expression of *P5CS1* in the absence of stress. Thus, our results provide further evidence for a previously unrecognized role for purine metabolites in stress responses, supporting the possible contribution of purine degradation to plant acclimation to changing environments.

Key words: Abiotic stress, allantoin, purine metabolism, stress response, uric acid.

The catabolism of organic nitrogen allows remobilization and recycling of nitrogen to sustain growth, development and reproduction of plants. Nucleobases, especially purine bases, provide a valuable source of remobilized nitrogen, since the complete breakdown of a purine ring, which proceeds through the intermediate formation of urate and the ureide allantoin, liberates four mole-equivalents of ammonium, which can be re-assimilated into amino acids (Figure 1A). When degraded, all purines, such as adenine, guanine and hypoxanthine, are converted to xanthine before heterocyclic ring opening and subsequent ureide hydrolysis (Moffatt and Ashihara 2002; Zrenner et al. 2006). Therefore, the oxidation of xanthine to urate is the bottleneck in the catabolic pathway, and the molybdo-flavoenzyme xanthine dehydrogenase (XDH; Figure 1A) catalyzes this critical step. To evaluate the physiological significance of purine catabolism, we used RNA interference-mediated gene silencing to knockdown simultaneously the expression of *AtXDH1* and *AtXDH2*, the two *Arabidopsis thaliana* (Arabidopsis) paralogs encoding XDH. Phenotypic characterization of severe *XDH*-knockdown mutants of Arabidopsis provided the first direct evidence for the importance of purine catabolism in plant growth and physiological processes such as leaf senescence (Nakagawa et al. 2007).

In plants, catabolism of purines is part of nitrogen metabolism; however, certain intermediate metabolites, such as allantoin, could also function in stress protection (Werner and Witte 2011). For example, reports in various plants describe the correlation between endogenous allantoin levels and physiological responses to drought (Alamillo et al. 2010; Yobi et al. 2013), high salinity (Kanani et al. 2010; Sagi et al. 1998), nutrient deprivation (Nikiforova et al. 2005; Rose et al. 2012), and pathogen infection (Montalbini 1991). The results obtained from examination of loss-of-function Arabidopsis mutants support the protective role of purine metabolites in plant tolerance to abiotic stress. Our *XDH*-knockdown mutants show significantly reduced tolerance to drought-

Abbreviations: ABA, abscisic acid; ALN, allantoinase; Arabidopsis, *Arabidopsis thaliana*; F_v/F_m, ratio between light-induced variable and maximum fluorescence of chlorophyll; P5CS1, Δ^1-pyrroline-5-carboxylate synthase 1; PDH1, proline dehydrogenase 1; qPCR, quantitative polymerase chain reaction; ROS, reactive oxygen species; WT, wild-type; XDH, xanthine dehydrogenase.
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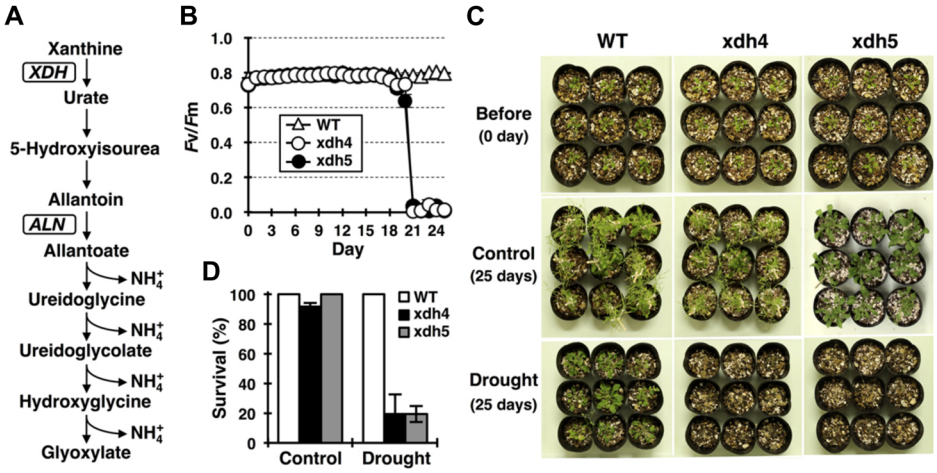

Figure 1. Sensitivity of Arabidopsis *XDH*-knockdown mutants to prolonged drought conditions. (A) The outline of purine degradation pathway, noting the two key enzymes xanthine dehydrogenase (XDH) and allantoinase (ALN). (B–D) Three-week-old plants of wild-type (WT) and *XDH*-knockdown mutant lines (xdh4 and xdh5) were subjected to progressive drought for 21 days by withholding water and then re-watered for a 4-day recovery. (B) Effects of drought on photosystem II activity (measured as F_v/F_m) in mature leaves. (C) Representative pictures of plants before being subjected to drought and after recovery. Control plants were kept watered throughout the treatments. (D) Survival rates. Three independent experiments were performed, with at least 12 plants for each line for each treatment in one experiment. The data are the mean±standard errors (SE).

shock stress (Watanabe et al. 2010) and knocking out *AtXDH1* alone enhances the sensitivity to extended darkness (Brychkova et al. 2008). The increased stress sensitivity of these *XDH*-impaired Arabidopsis might be attributable to the deficiency of certain purine metabolites because the application of exogenous urate or allantoin rescued the *XDH*-knockdown/knockout phenotype (Brychkova et al. 2008; Nakagawa et al. 2007; Watanabe et al. 2010). The role of such purine metabolites in plant stress protection remains to be elucidated. Treatment with exogenous allantoin can mitigate oxidative damage symptoms from a tissue to a whole-plant level (Brychkova et al. 2008; Gus'kov et al. 2004; Wang et al. 2012), suggesting its possible role in scavenging reactive oxygen species (ROS). However, allantoin per se seems not to possess antioxidant activity, indicating other possibilities for its protective action (Wang et al. 2012). Our recent reverse genetics study proposed a new and unpredicted role for this purine metabolite. We found that Arabidopsis mutants for *allantoinase* gene (*ALN*; see Figure 1A) defective in allantoin degradation showed enhanced drought and osmotic tolerance, which most likely resulted from an accumulation of allantoin, which primed stress responses by increasing the basal levels of the stress hormone abscisic acid (ABA) (Watanabe et al. 2014). Furthermore, both *ALN*-knockout mutation and exogenous allantoin resulted in coordinated activation of the biochemical pathways leading to ABA production, suggesting that allantoin might play a regulatory, rather than functional (i.e. antioxidant) role in stress protection, by activating ABA metabolism (Watanabe et al. 2014). To test this hypothesis and to further substantiate the possible regulatory role of purine metabolites, here we examined the effects of *XDH* knockdown on abiotic stress responses in Arabidopsis. The rationale behind this study was that if certain purine metabolites such as allantoin participate in the regulation of stress protection, the *XDH*-knockdown mutants would exhibit perturbed or impaired stress responses due to their inability to degrade xanthine into downstream metabolites (Figure 1A).

Previously, we demonstrated that *XDH*-knockdown mutants are highly susceptible to drought stress (Watanabe et al. 2010). However, we only evaluated the stress sensitivity of aseptically grown young seedlings exposed to air-drying conditions (often called "drought shock"). We therefore examined the effect of disrupted purine catabolism on Arabidopsis tolerance to prolonged drought stress similar to that encountered in field conditions. Wild-type (WT; C24 accession) plants and two independent *XDH*-knockdown lines (xdh4 and xdh5; Nakagawa et al. 2007) were grown for 2 weeks on sterile half-strength Murashige–Skoog (1/2MS) solid medium containing 0.3% (w/v) gellan gum and 1% (w/v) sucrose, pH 5.6, and then transferred to adequately irrigated pots containing a mixture of vermiculite and perlite (1 : 1, v/v). After one week of the transfer, progressive drought was induced by withholding water, during which plants were kept at 22°C under a 16-h photoperiod with a light intensity of 70 μmol photons m^{-2} s^{-1}. From the beginning of the drought treatment, we monitored changes in the maximum quantum yield of photosystem II (measured as F_v/F_m, the ratio between light-induced variable and maximum fluorescence of chlorophyll) of photosynthesis in mature rosette leaves using a pulse-amplitude modulated fluorometer (Junior-PAM, Walz GmbH,

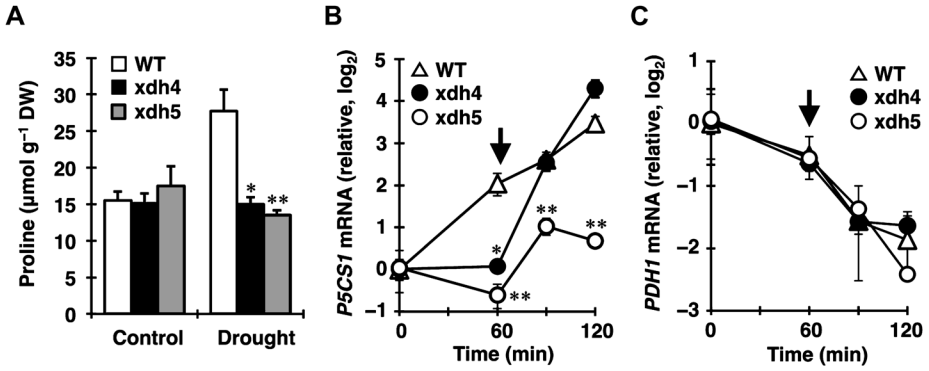

Figure 2. Compromised drought-stress responses of proline biosynthesis in Arabidopsis *XDH*-knockdown mutants. Aseptically grown 14-day-old seedlings from WT and *XDH*-knockdown lines (xdh4 and xdh5) were exposed to drought shock for 60 min, after which they were re-watered for a 60-min recovery. (A) Free proline levels before and after drought shock. (B, C) Relative mRNA levels of *P5CS1* (B) and *PDH1* (C) encoding the rate-limiting enzymes for proline biosynthesis and degradation, respectively, as monitored by qPCR using *ACTIN2* transcripts for calibration. A vertical arrow indicates the time at which plants were re-watered. The data are the means of three independent experiments±S.E.; $*p<0.05$, $**p<0.01$ (Student's *t*-test).

Effeltrich, Germany). We measured this parameter because a sudden decline of F_v/F_m reflects terminal water loss in Arabidopsis leaves under drought conditions (Woo et al. 2008). WT plants maintained high F_v/F_m values (close to 0.8), but the two knockdown mutants showed a rapid drop in F_v/F_m on the 21st day from the onset of stress treatment (Figure 1B). At this point, all plants were rehydrated and scored for the survival after a 4-day recovery period (Figure 1C). These assays revealed that prolonged drought drastically lowered the survival rates of the *XDH*-knockdown mutants, whereas WT plants exhibited little mortality (Figure 1D). Under the control conditions, both WT and knockdown lines showed similar survival rates. These observations, combined with our previous drought-shock experiments (Watanabe et al. 2010), indicate that purine catabolism is involved in general drought tolerance in Arabidopsis.

Next, we attempted to elucidate the mechanism underlying the hypersensitivity to drought stress of the *XDH*-knockdown mutants. One of the ubiquitous responses in plants to abiotic stress, including drought, is the accumulation of compatible solutes such as proline. Under stress conditions, free proline accumulation is partly mediated by both transcriptional up-regulation of proline-biosynthetic enzymes and down-regulation of proline-degrading enzymes (Yoshiba et al. 1997). We therefore compared changes in the levels of free proline and mRNAs for proline metabolism-related genes between WT and knockdown lines in response to drought shock. Aseptically grown 14-day-old plants were exposed to open-air conditions for 60 min, after which they were re-watered for 60 min and then harvested for proline determination and RNA extraction, as described in detail in Watanabe et al. (2010). Quantitative polymerase chain reaction (qPCR) was performed as described (Watanabe et al. 2014), with reverse-transcribed RNA to quantify mRNAs for *Δ[1]-pyrroline-5-carboxylate synthase 1* gene (*P5CS1*) and *proline dehydrogenase 1* gene (*PDH1*), which encode the key enzymes in proline biosynthesis and degradation, respectively. *ACTIN2* transcripts were used as a reference. PCR primers were as follows: 5′-TGG AAG ATT GGC TCT TGG TC-3′ (forward) and 5′-GTC GAT AAC GAA GCC TTT GC-3′ (reverse) for *P5CS1*; 5′-ATG CGG AAG ACA CAA TCC TC-3′ (forward) and 5′-TGC CAA ATG CAG TCT CTC AC-3′ (reverse) for *PDH1*; 5′-ACC GTA TGA GCA AAG AAA TCA C-3′ (forward) and 5′-GAG GGA AGC AAG AAT GGA AC-3′ (reverse) for *ACTIN2*. When exposed to drought shock, free proline increased 2-fold in WT plants, but remained unchanged in *XDH*-knockdown mutants (Figure 2A). Consistent with the difference in proline levels, WT plants accumulated *P5CS1* transcripts during the stress treatment (i.e. the first 60 min in Figure 2B), whereas the knockdown mutants showed reduced or delayed induction of *P5CS1* expression. By contrast, *PDH1* mRNA levels showed a similar pattern of transcriptional down-regulation in both WT and knockdown lines (Figure 2C). These observations suggest that *XDH* suppression, and hence purine catabolism dysfunction, might perturb drought-induced proline biosynthesis by affecting the transcription of the key biosynthetic enzyme.

Disrupted at an early step in the purine-catabolic pathway, the *XDH*-knockdown mutants exhibit significantly lower levels of the downstream metabolites, such as urate, than WT plants (Watanabe et al. 2010). Because the purine metabolite deficiency occurs with the compromised stress response as shown above (Figure 2A, 2B), and because the intermediary metabolite allantoin can evoke stress-responsive gene expression (Watanabe et al. 2014), we examined the possible enhancing effect of urate and allantoin on *P5CS1* expression in Arabidopsis. Sterilized seeds of WT plants were germinated and grown at 22°C in liquid culture consisting of 1/2MS

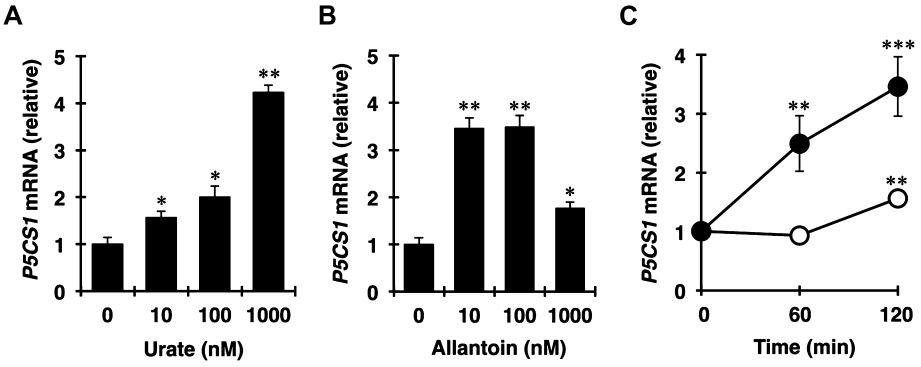

Figure 3. Induction of *P5CS1* transcripts by exogenously supplied purine metabolites in Arabidopsis. Hydroponically grown, 12-day-old WT seedlings were treated with urate or allantoin and *P5CS1* mRNA levels were determined by qPCR. (A, B) Dose-dependent responses of *P5CS1* expression upon treatments with urate (A) and allantoin (B) at indicated concentrations for 120 min. (C) Time-course *P5CS1* expression profiles in response to 10 nM urate (open circle) or allantoin (closed circle). The data are the means of three independent experiments±S.E.; * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (Student's *t*-test).

basal salts and 1% sucrose, pH 5.6, under continuous light with a rotation speed of 120 rpm. After 12 days, hydroponically grown seedlings were treated with various concentrations (10 to 1000 nM) of urate or allantoin for 120 min and the levels of *P5CS1* transcripts were quantified by qPCR. Both purine metabolites increased *P5CS1* expression at a similar level with different dose dependencies (Figure 3A, 3B). In the range of concentrations used in our experiments, urate enhanced the levels of *P5CS1* transcripts by up to 4.2-fold in a dose-dependent manner while the effective allantoin concentration peaked between 10 and 100 nM resulting in 3.5-fold induction of *P5CS1* expression. Time-course experiments revealed that, at the same concentration (10 nM), allantoin produced an appreciable stimulatory effect in a shorter incubation time than urate (within 60 min, Figure 3C). Since urate is the precursor to allantoin (Figure 1A), it is probable that the observed effect of urate may result from its catabolism to allantoin.

Although exogenous supplementation of urate and allantoin resulted in increased transcript levels of *P5CS1*, it remains unclear whether these purine metabolites could mediate the up-regulated expression directly or indirectly. Recently, we found that allantoin induces the expression of typical stress-responsive genes, such as *RD29A*, *RD29B* and *RD26*, in Arabidopsis seedlings under normal growth conditions (Watanabe et al. 2014). The observed induction of these genes appears to depend on ABA, as allantoin can increase free ABA levels by activating the rate-limiting steps in both the de novo biosynthesis and deconjugation of inactive ABA-glucose ester, but mutations in either ABA-generating pathway abrogate the effect of allantoin on stress-responsive gene expression (Watanabe et al. 2014). In Arabidopsis, *P5CS1* is also induced by abiotic stress and ABA (Strizhov et al. 1997; Yoshiba et al. 1995). These observations suggest that the effect of the purine metabolites on *P5CS1* induction is more likely to be indirect, possibly through the mechanism involving ABA, although this needs experimental confirmation. In this study, exogenous allantoin effectively induced expression of *P5CS1* at lower concentrations than were needed to elicit expression of the aforementioned stress-responsive genes in the previous study (Watanabe et al. 2014). This difference probably arises from the different culture systems used to administer allantoin (i.e. liquid versus solid medium), as hydroponically grown plants can take up nutrients and other components much more efficiently from the culture medium. Upon cellular uptake, the two purine metabolites are ultimately metabolized to release ammonium (Figure 1A), the primary form of assimilable nitrogen also known to mediate signal transduction and affect global gene expression in plants (Patterson et al. 2010). In our experiments, however, it is unlikely that ammonium derived from exogenously added urate or allantoin acts as such a signal because the culture medium is composed of much higher concentrations of this inorganic nitrogen.

Overall, the above observations suggest that the compromised stress response may at least partially account for the drought-hypersensitive phenotype of *XDH*-knockdown mutants. We reasoned that if this were indeed the case, these mutants would also exhibit higher susceptibility to other kinds of stress than WT plants. Various stress conditions involve the generation of ROS and ROS-induced oxidative damages in plants (Elstner 1991). We therefore tested the sensitivity of *XDH*-knockdown mutants to ROS-mediated oxidative stress using root growth assays (Rizhsky et al. 2004). Surface-sterilized seeds were placed on square Petri plates containing 1/2MS basal salts, 0.3% gellan gum, 1% sucrose and different concentrations of superoxide-generating paraquat. The plates were incubated at 4°C for 2 days in the dark, and then placed vertically at an angle of 80° in a growth cabinet at 22°C with a 16-h photoperiod (60 μmol photons m^{-2} s^{-1}). After 7 days,

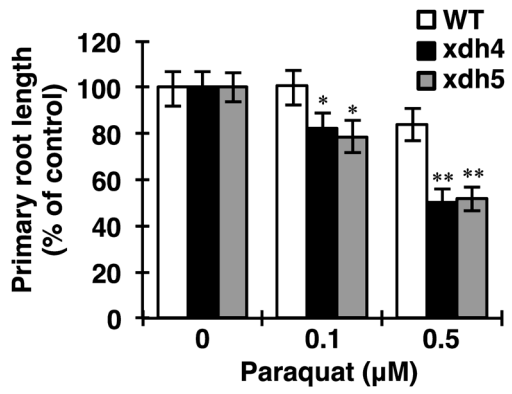

Figure 4. Sensitivity of Arabidopsis *XDH*-knockdown mutants to ROS-mediated oxidative stress. Taproot elongation was evaluated after seed germination and plants were grown for 7 days under a 16-h illumination period on medium containing the superoxide-generating chemical paraquat. Three independent experiments were performed, with at least 10 plants for each line for each treatment in one experiment. The data are the mean±S.E.; * $p<0.05$, ** $p<0.01$ (Student's *t*-test).

the knockdown mutants showed significantly reduced primary root elongation in the presence of paraquat, indicating that they are more susceptible to oxidative stress than WT plants (Figure 4). These results, while not providing mechanistic details, further support the protective role of purine metabolites in plant responses to stress.

In conclusion, we showed that Arabidopsis becomes highly vulnerable to progressive drought and oxidative stress in the absence of functional purine degradation, which may result from compromised stress responses, as exemplified by defective proline accumulation and perturbed expression of the key biosynthetic enzyme. The results from this work and our previous finding that allantoin enhances abiotic stress tolerance (Watanabe et al. 2014) complementarily point to a previously unrecognized contribution of purine metabolism in plant stress protection, possibly through the stimulatory action of the intermediary metabolites on the stress responses.

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