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Adverse effect of urease on salt stress during seed germination in *Arabidopsis thaliana*

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ABSTRACT

Seed germination is a critical stage in the development of crops that grow in saline soils. We noticed that seeds of an *Arabidopsis* urease mutant have significantly increased salt stress tolerance. To understand why, we treated the wild type (WT) with a urease inhibitor and found that its salt stress tolerance was also improved. We hypothesized that urease acting on urea generates NH_4^+, which probably exacerbates salt stress. As expected, the urease inhibitor significantly decreased the NH_4^+ level in WT seeds. These findings suggest that blocking urease activity improves salt tolerance during seed germination by lowering the concentration of NH_4^+.

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1. Introduction

Salt stress is a major abiotic stress that inhibits crop growth [1]. More than 20% of cultivated land worldwide (about 45 million hectares) is affected by salt stress and the amount is increasing day by day [2–4]. High salinity causes both ionic and osmotic stresses that, in turn, lead to secondary stresses such as oxidative stress and nutritional disorders [5,6]. Seed germination is the most critical stage for survival during the life of a plant in saline environments. An improved understanding of molecular responses to NaCl treatment on seed germination may therefore facilitate the development of crops with increased tolerance to NaCl stress.

Salt tolerance in different plant species has been linked to a large number of genes and transcription factors, many of which are classified in the functional categories of ion transport or homeostasis (e.g., SOS, NHX1, HKT1 and H^+-ATPase), senescence-associated genes (e.g., SAG), molecular chaperones (e.g., HSP genes), and dehydration-related transcription factors (e.g., DREB), etc. [7–20]. Urease (EC 3.5.1.5) appears to be another salt stress-related factor as in preliminary studies we found that an *Arabidopsis thaliana* urease deficiency mutant (*aturease*) has increased tolerance to salt stress during seed germination.

Ureases are nickel-dependent metalloenzymes that catalyze the conversion of urea to ammonia and carbamate, which spontaneously decomposes into carbonic acid and another molecule of ammonia [21]. These enzymes are widespread in nature, being present in plants, bacteria, and fungi. Some plants, such as soybean (*Glycine max*) have two urease genes [22,23], while others, including potato (*Solanum tuberosum*), tomato (*Lycopersicon esculentum*) and Arabidopsis (*A. thaliana*; ecotype Columbia [Col]) have only one [24]. In plants, ureases metabolize urea that arise from different pathways [25–29]. One such pathway during salt stress involves the degradation of storage proteins during seed germination, which increases the arginine content [30]. Arginase (EC 3.5.3.1) acting on the arginine generates urea [31]. Although urease

Abbreviations: PPD, phenyl phosphorodiamidate; 1/2 MS, one-half-strength Murashige and Skoog; 1/2 MS-N, half concentration of regular MS without vitamins, sugars, amino acids, or ammonium nitrate and potassium nitrate

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plays an essential role involved in urea metabolism, the relationship between the urea metabolism and salt tolerance during seed germination has not been yet investigated.

In this study, to elucidate the relation between urea metabolism and salt tolerance, we analyzed the effect of salt stress on the growth of *aturease*, and the effect of a urease inhibitor (phenyl phosphorodiamidate, PPD) on the growth and NH_4^+ concentration of WT plants. Our results suggest that blocking urease activity improved salt tolerance during seed germination, probably by lowering the concentration of NH_4^+ derived from urea metabolism.

2. Materials and methods

2.1. Plant materials and growth conditions

A. thaliana (ecotype Col-0) and a T-DNA-disrupted *AtUrease* mutant (*aturease*; SALK_038002) were used in this study. The latter was obtained from the Arabidopsis Biological Research Center (ABRC). Seeds were surface-sterilized and plated on five different types of solid one-half-strength Murashige and Skoog (1/2 MS) medium, in which cobalt chloride was replaced with 0.5 μM nickel chloride [32]: (1) 1/2 MS (i.e., half concentration of regular MS); (2) 1/2 MS-N (i.e., half concentration of regular MS without vitamins, sugars, or amino acids, ammonium nitrate and potassium nitrate were omitted, and 9.35 mM KCl was added to maintain the same molarity of potassium ions); (3) 1/2 MS + NaCl (0, 75, 125, 135, 150 mM) and Na_2SO_4 (0, 50, 75 mM); (4) 1/2 MS + urea (0 and 10 mM); (5) 1/2 MS-N + 9.35 mM KNO_3 or 4.68 mM urea.

To reduce variation in germination, we sorted the seeds with an 80-mesh sieve to remove smaller seeds, and used seeds between 250 and 300 μm in size. Germination was defined as an obvious emergence of the radical through the seed coat. Cotyledon greening was defined as obvious cotyledon expansion and turning green. After 2 d of cold (4 °C) treatment, the seedlings were grown at 22 °C under a 12-h-light/12-h-dark cycle in a growth chamber. The seed germination percentages were evaluated every day during the germination tests. Root length and fresh weight were measured after 2–3 weeks cultivation. Three biological replications were used for statistical analyses. The significance of differences between groups was analyzed with Student's *t*-tests.

2.2. Mutant characterization

Homozygous lines of the *aturease* mutant were identified using PCR amplification and RNA gel blotting. Gene-specific primers were used, respectively: for *aturease*, primer LP + primer RP (GACAGCTGACAAGATGAAGGC; AACAACATTGGTCCTTGCAAC); for actin, primer actin-FW + primer actin-RV (GAAAATGGCTGATGGT GAAG; CATAGATAGGAACAGTGTGG). The homozygous lines of the mutant were screened with a primer binding at the left border of the T-DNA insert (LBP: ATTTTGCCGATTTCGGAAC for the Salk Institute collection) in combination with a gene-specific primer. For northern blot analysis, total RNA was obtained using Trizol. Denaturing gelelectrophoresis was carried out to examine the *AtUrease* transcript levels in the *aturease* mutant homozygous lines, which was marked with a probe labeled with digoxigenin (DIG, Roche, USA) with RNA gel blot analysis, following the methods of Guo et al. [33]. Signals were detected using a luminescent image analyzer (Fijifilm, LAS-4000mini, Japan).

2.3. Effect of urease inhibitor on seed germination

Seeds of *Arabidopsis* were sown on filter papers soaked with modified 1/2 MS liquid media with 135 mM NaCl and supplemented with different concentration of PPD (0, 3, 7.5, and 15 μM). The percentage of green cotyledons and the root length were measured after 10 days cultivation.

2.4. Ammonium concentration assay

Cotyledons and roots of WT plants were gently washed with ice-cold Milli-Q water, wiped dry with tissues, and immediately frozen in liquid N2. Half a gram of frozen tissue, 5 ml ice-cold extraction medium and a small amount of quartz sand were placed in a chilled mortar. The tissue was then ground to a fine powder with a chilled pestle. The plant tissue was extracted with 450 μL of 80% (v/v) ethanol, heated to 80 °C for 20 min, mixed with 500 μL water, heated to 80 °C for 20 min and centrifuged at 25 000×*g* at 2 °C for 10 min. The following 4 solutions were prepared: (I) phenol–ethanol solution: 11 mM phenol in 95% (v/v) ethanol; (II) Nitroprusside solution: 1.7 mM sodium nitroprusside in a dark bottle and stored for no longer than one month; (III) Alkaline solution: 0.68 M trisodium citrate in 0.25 M NaOH solution; (IV) Oxidizing solution: 16 ml of solution III was mixed with 0.4 ml 2 M sodium hypochlorite and 3.6 ml water. The supernatant (800 μL) was mixed with 100 μl of solution I, 100 μl solution II and 250 μl of solution IV. pH was checked and the color was allowed to develop in the dark for 60 min at 25 °C. Finally, NH_4^+ content was determined colorimetrically at 640 nm.

3. Results and discussion

3.1. Characterization of AtUrease knockout mutant

The T-DNA insertion is located in the last exon of *AtUrease* (Fig. 1A), as confirmed by PCR-based genotype analyses (Fig. 1B). RT-PCR and Northern blotting analyses of the homozygous T-DNA insertion *aturease* mutants show that they completely lack *AtUrease* transcripts (Fig. 1B). *aturease* mutant plants were unable to grow on urea as the sole nitrogen source (Fig. 1C). In contrast, wild-type accessions grew well with either nitrate or urea (Fig. 1C). This experiment was repeated 3 times with identical results. These results, which are consistent with a previous study [24], indicate that the homozygous T-DNA insertion in the *AtUrease* locus resulted in a null mutant.

The seeds of WT and *aturease* were germinated on 1/2 MS medium, which has sufficient nitrogen with and without 10 mM urea. WT plants grew significantly less in 10 mM urea than in 0 mM urea (Fig. 1D–G), consistent with previous findings that urea fertilizer has adverse effects on seed germination, seedling growth, and early plant growth [34,35]. However, the root length of *aturease* plants were longer in 10 mM urea than in 0 mM urea (Fig. 1D–F), meanwhile, the root lengths (Fig. 1F) and fresh weight (Fig. 1G) of *aturease* plants were higher than those of WT plants after 10 mM urea treatment. These results suggest that loss of function of AtUrease can alleviate the toxicity derived from additional urea.

3.2. Loss of function of AtUrease enhances salt tolerance during seed germination

After seed stratification on 1/2 MS medium supplemented with different concentrations of NaCl (Fig. 2B–D), the seeds of *aturease* mutants germinated and grew better than WT seeds. On the other hand, the growth rates of *aturease* and WT were not obviously different in medium without salt stress (Fig. 2A). Moreover, in the presence of different concentrations of NaCl (125, 135 and 150 mM), the root lengths and fresh weights of *aturease* mutants were markedly higher than those of WT plants (Fig. 2E–F). As salt stress increased, it had little effect on the germination of *aturease* seeds, whereas it increasingly inhibited germination of WT seeds

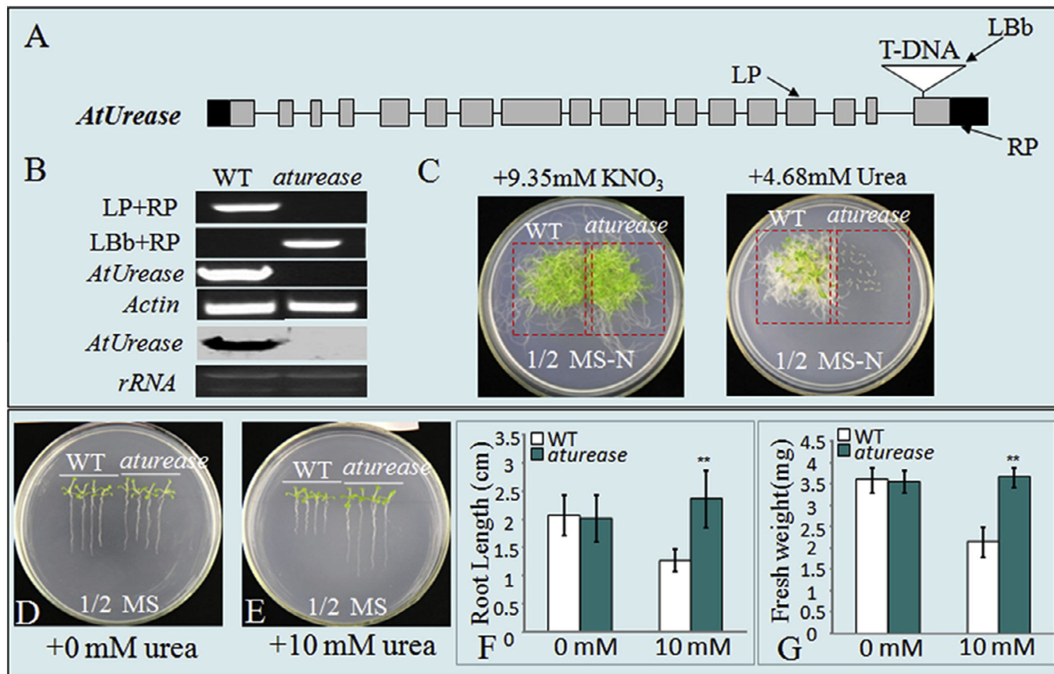

Fig. 1. Characterization of *aturease*, an Arabidopsis T-DNA insertion mutant. (A) T-DNA insertion site in *urease*; gray boxes represent exons; black lines represent introns; (B) Reverse-transcription PCR (RT-PCR) and Northern blotting analysis to confirm the knockout status of *aturease*; *Actin* expression and *rRNA* were used as the internal control for RT-PCR and Northern blotting, respectively. (C) Growth test on agar plates with nitrate or urea as nitrogen source; left column, plates supplemented with nitrate; right column, plates supplemented with urea. Photographs were taken 4 weeks after germination; (D–E) Wild-type (WT) and *aturease* seeds germinated on 1/2 MS medium supplementary 0 and 10 mM urea. Photographs (D–E) were taken 2 weeks after germination; (F–G) the root lengths and fresh weight of WT and *aturease* plants were measured after the treatments (D–E). Each value represents the means ± S.E.s of 15 plants. Statistical significance was determined using the Student's *t*-test. * Represents $p < 0.05$ and ** represents $p < 0.01$.

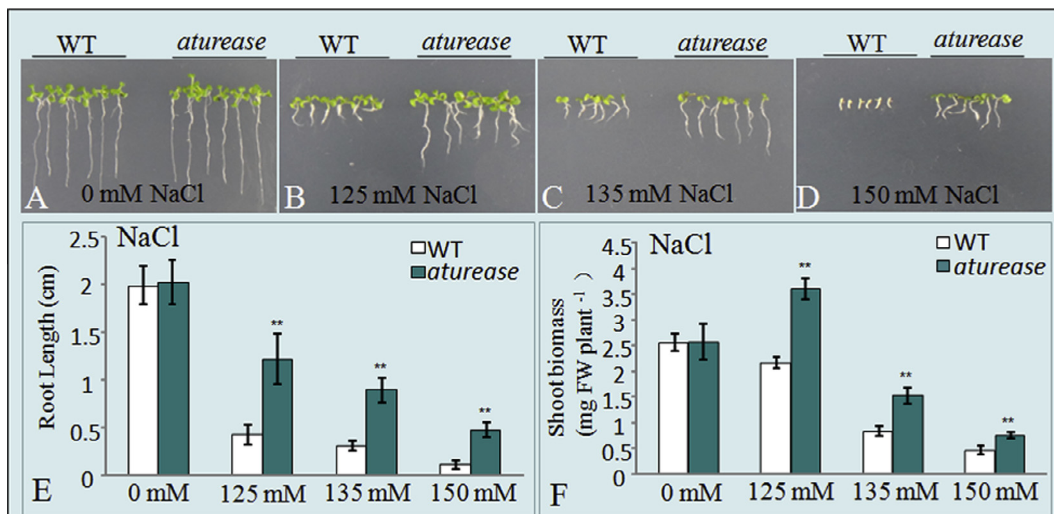

Fig. 2. Salt stress tolerance of wild-type (WT) and *aturease* plants. Seeds germinated on half-strength MS medium (A) containing 125 (B), 135 (C) and 150 (D) mM NaCl. Photographs were taken 3 weeks after germination; (E and F) The root lengths and fresh weight of WT and *aturease* plants were measured after the indicated treatments. Each value represents the means ± S.E.s of 15 plants. Statistical significance was determined using the Student's *t*-test. * Represents $p < 0.05$ and ** represents $p < 0.01$.

(Fig. 3). The difference was most pronounced on the second day after germination. These results demonstrate that a loss of function of AtUrease enhances salt tolerance during seed germination. After seeds stratification on 1/2 MS medium without NaCl for 12, 24, 48, or 72 h, then transferred to NaCl containing medium to investigate the phenotype of WT and *aturease*. Interestingly, when *aturease* seeds were transferred to NaCl-containing medium after 48 h stratification without NaCl, the root lengths of *aturease* mutants were not markedly higher than those of WT plants (Supplemental Fig. S2). However, seeds of an *Arabidopsis* urease mutant have increased salt stress tolerance 24 h before transferring. These results suggest that AtUrease was involved in NaCl responses during the early seed-germination stage.

3.3. Effect of ammonium on seed germination under salt stress

PPD has been used as an inhibitor of AtUrease activity in Arabidopsis and other plants [34,36]. Germination of WT seeds

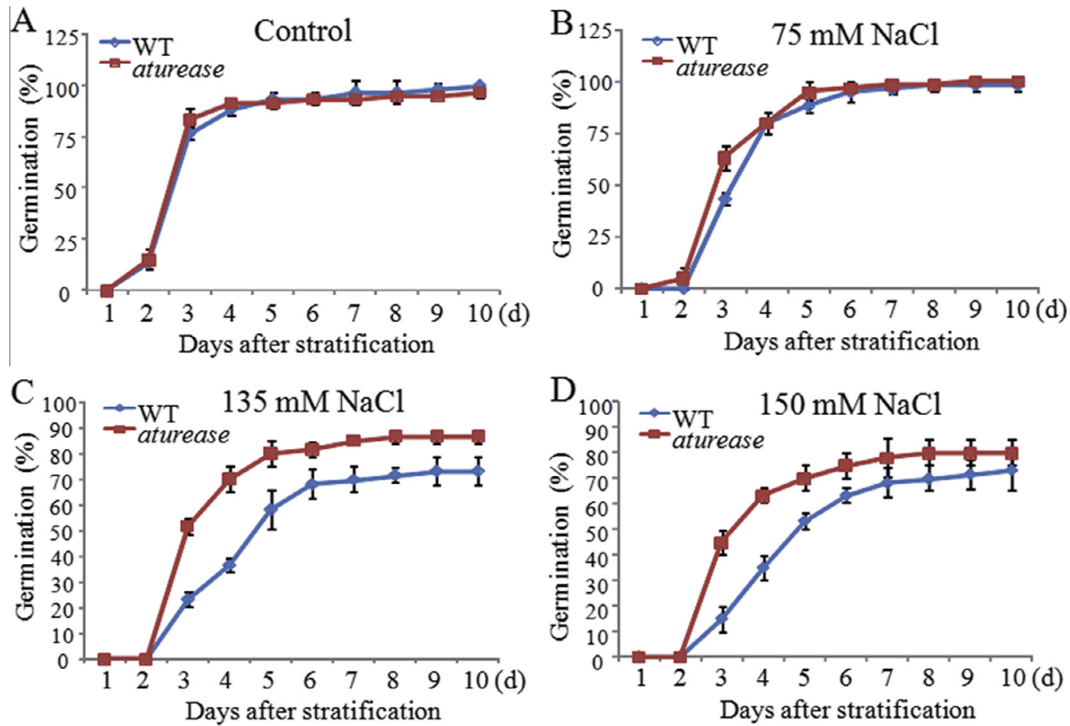

Fig. 3. NaCl responses of Wild-type (WT) and *aturease* germinated seeds: germination rates on half-strength MS medium containing 0 (A), 75 (B), 135 (C) or 150 (D) mM NaCl during the time course, respectively. Data show the mean ± S.D. of at least three replicates; at least 35 seeds per genotype were counted in each replicate.

was clearly improved by increasing the concentration of PPD in the salt medium (Fig. 4B–E), whereas the seeds germinated well in the control medium (Fig. 4A). In the absence of PPD, the cotyledon-greening percentage was 90% in the absence of NaCl but less than 10% in the presence of 135 mM NaCl. However, in the presence of PPD and 135 mM NaCl, the cotyledon-greening percentage gradually increased with increasing PPD concentration, peaking at 15 μM PPD (Fig. 4F). The root lengths of the seedlings were significantly shorter in the presence of 135 mM NaCl than in the absence of NaCl and PPD. However, the root lengths increased with increasing PPD concentration in the NaCl medium (Fig. 4G). These results indicate that inhibiting urease activity can reduce the toxicity of salt stress during seed germination. The phenotype of WT plants in the presence of PPD and NaCl is similar to that of *aturease* under salt stress (Fig. 2). In addition, previous studies have shown that Arabidopsis urease accessory proteins AtUreD, AtUreF, and AtUreG are required in vivo to generate a functional urease enzyme, no urease activity was detected in any of the mutants [37], thus the phenotype of *atureD* mutant under salt stress was analyzed, the results show that the root lengths and fresh weights

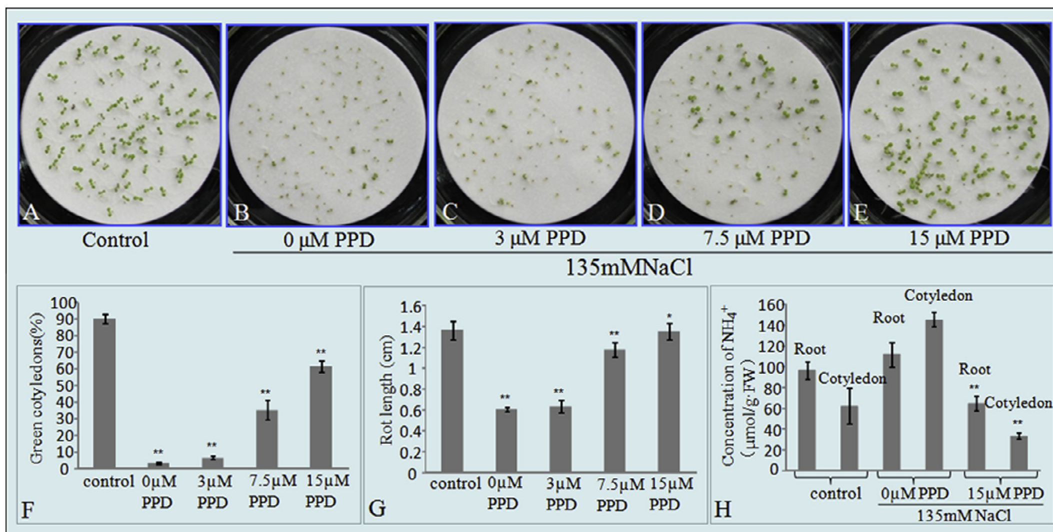

Fig. 4. Effect of AtUrease inhibitor PPD on germination of Wild-type (WT) and *aturease* seeds: (A–E) germinated on half-strength MS or 135 mM NaCl with or without 3, 7.5 or 15 μM PPD. Representative images show the morphology of seedlings 10 d after stratification. (F–H) Green cotyledons, root length and NH_4^+ concentration were determined 10 d after the stratification. Data are means and S.D. from three biological replicates each with at least 60 seeds. Statistical significance was determined using the Student's *t*-test. * Represents $p < 0.05$ and ** represents $p < 0.01$.

of *atureD* mutants were markedly higher than those of WT plants in the presence of 135 mM NaCl (Supplemental Fig. S1). The results for *atureD* also provide evidence that blocking urease activity improves salt tolerance during seed germination.

Stimulated salt stress during seed germination causes the degradation of a large number of storage proteins, which significantly increases the arginine content, which, in turn, leads to the generation of urea by arginase, AtUrease hydrolyzes urea to CO_2 and ammonia (or NH_4^+) [31], moreover, high levels of NH_4^+ in plant cells can inhibit plant growth [38–40]. In this study, WT plants were more sensitive to NaCl than *aturease*. We speculated that seed germination was inhibited by salt stress probably due to the generation of ammonia (or NH_4^+) from urea metabolism.

In the presence of 135 mM NaCl, NH_4^+ concentrations of both root and cotyledon were clearly higher than in the controls (without NaCl and PPD) (Fig. 4H). However, adding 15 μM PPD to the 135 mM NaCl medium significantly reduced NH_4^+ concentration of the root and cotyledon during seed germination, particularly in the root (Fig. 4H). These results suggest that reducing urease activity by PPD to lower the concentration of NH_4^+ in the root and cotyledon of WT plants alleviates the NH_4^+ toxicity under salt stress conditions. Similarly, excessive exogenous urea inhibited growth of the WT plants (Fig. 1D–G), probably due to the NH_4^+ derived from the hydrolysis of urea. In addition, the *aturease* mutants were more tolerant to salt stress than WT plants during germination (Figs. 2 and 3), probably because the blocking of urea metabolism prevented the generation of NH_4^+, which improved seed germination under salt stress. Although plant ureases are essential enzymes for urea metabolism, high activity of urease has a harmful effect on seed germination under salt stress conditions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.04.016.

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