

**Regular Paper** 

# A Role for Allantoate Amidohydrolase (AtAAH) in the Germination of Arabidopsis thaliana Seeds

Farzaneh Yazdanpanah<sup>1,2</sup>, Leo A.J. Willems<sup>1</sup>, Hanzi He<sup>1,3</sup>, Henk W.M. Hilhorst<sup>1</sup> and Leónie Bentsink<sup>1,\*</sup>

<sup>1</sup>Wageningen Seed Science Centre, Laboratory of Plant Physiology, Wageningen University, Wageningen 6708 PB, The Netherlands

<sup>2</sup>Present address: Department of Cell & Molecular Biology, Faculty of Life Sciences & Biotechnology, Shahid Beheshti University, G.C., Evin, Tehran 1983963113, Iran.

<sup>3</sup>Present address: College of Plant Science and Technology, Huazhong Agricultural University, Wuhan 430070, Hubei, China.

\*Corresponding author: E-mail, leonie.bentsink@wur.nl

(Received 6 August 2021; Accepted 20 July 2022)

Seed dormancy is a very complex trait controlled by interactions between genetic and environmental factors. Nitrate is inversely correlated with seed dormancy in Arabidopsis. This is explained by the fact that seed dry storage (after-ripening) reduces the need for nitrogen for germination. When nitrate is absorbed by plants, it is first reduced to nitrite and then to ammonium for incorporation into amino acids, nucleic acids and chlorophyll. Previously, we showed that ALLANTOATE AMIDOHYDROLASE (AtAAH) transcripts are up-regulated in imbibed dormant seeds compared with after-ripened seeds. AAH is an enzyme in the uric acid catabolic pathway which catalyzes the hydrolysis of allantoate to yield  $CO_2$ , NH<sub>3</sub> and S-ureidoglycine. This pathway is the final stage of purine catabolism, and functions in plants and some bacteria to provide nitrogen, particularly when other nitrogen sources are depleted. Ataah mutant seeds are more dormant and accumulate high levels of allantoate, allantoin and urea, whereas energy-related metabolites and several amino acids are lower upon seed imbibition in comparison with Columbia-0. AtAAH expression could be detected during the early stages of seed development, with a transient increase around 8 d after pollination. AtAAH expression is the highest in mature pollen. The application of exogenous potassium nitrate can partly complement the higher dormancy phenotype of the Ataah mutant seeds, whereas other nitrogen sources cannot. Our results indicate that potassium nitrate does not specifically overcome the alleviated dormancy levels in Ataah mutant seeds, but promotes germination in general. Possible pathways by which AtAAH affects seed germination are discussed.

**Keywords:** Ammonia • Arabidopsis thaliana • Block of germination • Nitrogen • Purine pathway • Seed dormancy

#### Introduction

Seed dormancy is an adaptive trait that regulates the timing of seed germination. Environmental factors, including light (i.e. light quality and photoperiod), temperature, water, nutrients, the duration of seed storage as well as growth conditions of the mother plant, influence the level of seed dormancy (Bewley 1997, Bewley et al. 2013). Nitrogen, which is essential for plant growth, development and reproduction, is inversely correlated with seed dormancy in Arabidopsis. Conditions that favor nitrate accumulation in mother plants and seeds lead to lower seed dormancy levels. Alboresi et al. (2005) showed that this effect is mediated by nitrate signaling and not the nutritional contribution of nitrate. Nitrate accumulation in seeds reduces the gibberellic acid requirement for germination and accelerates the decrease in ABA levels during early seed imbibition (Alboresi et al. 2005). It was shown that nitrate may act on seed dormancy, at least in part, via the production of nitric oxide (NO) and its subsequent effects on levels of ABA in seeds (Arc et al. 2013).

Seed dry storage, also referred to as after-ripening, leads to a reduction of seed dormancy. After-ripening can also be considered as a widening of the germination window, allowing seeds to germinate in conditions that inhibit the germination of freshly harvested seeds (Finch-Savage and Leubner-Metzger 2006, Soppe and Bentsink 2020). As a result, after-ripening also reduces the nitrogen requirement for germination (Finch-Savage et al. 2007).

Plants can take up nitrogen from the soil. Generally, they take it up in the form of ammonium or amino acids, but nitrate is preferred by plants that are adapted to higher pH and more aerobic soils (for a review, see Maathuis 2009). After absorbance, nitrate is usually first reduced to nitrite and then to ammonium before it is incorporated into amino acids, nucleic acids and chlorophyll (Smil 2000). Moreover, the nitrogen that is incorporated in pyrimidine and purine bases in the DNA and RNA of senescing leaves can be remobilized, and as such serve as a nitrogen source for the production of leaves and seeds (Malagoli et al. 2005, Diaz et al. 2008). The metabolic pathways required for this remobilization is largely conserved between plants, fungi and bacteria, involves a series of enzymatic steps and takes place in

© The Author(s) 2022. Published by Oxford University Press on behalf of Japanese Society of Plant Physiologists.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited.

Plant Cell Physiol. 63(9): 1298-1308 (2022) doi:https://doi.org/10.1093/pcp/pcac103, Advance Access publication on 21 July 2022, available online at https://academic.oup.com/pcp





Fig. 1 The S-allantoin degradation pathway and the effect of a defective AtAAH enzyme on Arabidopsis seed phenotypes. (A) Schematic presentation of S-allantoin degradation in plants. Conversions in the pathway and the enzymes responsible are indicated. (B) Open silique of the Ataah mutant in comparison with Columbia-0 (Col-0). The mutant contains several defective seeds indicated by the arrows. (C) Seed dormancy levels expressed as days of seed dry storage to reach 50% of germination (DSDS50) of Ataah, and of Col-0 and two independent Ataah complementation lines. (D) Seed performance of after-ripened seeds (germination after artificial aging and germination in stress conditions including: salt 130 mM; mannitol (-1 MPa) and ABA ( $0.15 \mu$ M)). Shown are averages of four biological replicates and their SE. Significant differences between Ataah and Col-0 are indicated (\*P < 0.05 and \*\*P < 0.01).

the cytosol, peroxisome and the endoplasmic reticulum (Vogels and Van der Drift 1976, Tipton 2006). Purine catabolism leads to allantoin and allantoate, compounds that have a favorable N:C ratio to serve nitrogen transport and storage. In plants, the first step of the ureide (allantoin and allantoate) metabolic pathway occurs in the cytoplasm and leads to xanthine. The next step, which is the oxidation of xanthine to uric acid, occurs in the cytoplasm. After this, uric acid is transported into the peroxisome and converted into S-allantoin by three enzymatic steps (Ramazzina 2006). The hydrolysis of S-allantoin by ALLAN-TOINASE (ALN) to allantoate and the breakdown of allantoate by ALLANTOATE AMIDOHYDROLASE (AAH) to usable nitrogen occur in the endoplasmic reticulum (Hanks et al. 1981) (Fig. 1A). Arabidopsis plants defective in AAH prevent the remobilization of purine nitrogen and are not able to grow when only allantoin is provided as the nitrogen source (Werner et al. 2008).

Possible involvement of the allantoate pathway in seed dormancy was suggested from studies on near isogenic lines that contain *DELAY OF GERMINATION* loci (*DOG* NILs). Seeds of these lines which displayed increased dormancy levels, induced by growing plants in low nitrate and low temperature conditions, have reduced seed allantoin and urea contents when compared with seeds developed in control conditions (He et al. 2016). Transcriptome analyses on the same seeds showed an up-regulation of ALN gene expression (He et al., 2016), which is probably a feedback reaction caused by the lack of ammonia. Moreover, it is known that ABA is required for the induction of seed dormancy (Hilhorst 1995) and that there is a link between purine catabolism and ABA. Studies using Arabidopsis mutants defective in purine catabolism have shown that the intermediary metabolite allantoin stimulated ABA production and enhanced abiotic stress tolerance (Watanabe et al. 2014a, Watanabe et al. 2014b). Furthermore, *Ataln* mutant seeds displayed deep dormancy relative to wild-type seeds, identifying AtALN as a negative regulator of seed dormancy (Piskurewicz et al. 2016).

We have identified AtAAH transcripts to be up-regulated in imbibed dormant seeds (Yazdanpanah et al. 2017). Here we investigated whether AtAAH plays a role in seed dormancy. Interestingly, seeds of the Arabidopsis loss-of-function Ataah mutant showed increased seed dormancy compared with wild-type Columbia-0 (Col-0). This dormancy could be partly compensated by the application of exogenous potassium



nitrate, either during the growth of the mother plant or during imbibition of the seeds.

#### Results

## Loss of function of *AtAAH* leads to partially defective seed maturation and increased seed dormancy

Homozygous T-DNA knock-out plants of AtAAH (At4G20070) grow normally. However, lack of functional AtAAH leads to a certain amount of seed abortion (Fig. 1B). A possible role for AtAAH during seed development is underlined by its high expression in pollen (http://bar.utoronto.ca/efp/cgi-bin/efp-Web.cgi) (Supplementary Fig. S1). Ataah mutant seeds display increased dormancy, expressed as a higher after-ripening requirement (DSDS50) and a higher sensitivity to salt (NaCl) and mannitol. The mutation did not affect seed longevity (germination after artificial aging) but the seeds were more tolerant to ABA (Fig. 1D). The similar pattern between salt and mannitol indicates that the inhibition of germination is likely to be an osmotic effect. The dormancy phenotype of the mutant is complemented by the introduction of wild-type AtAAH under a 35S promoter into the Ataah mutant background (Fig. 1C). This confirmed that the observed phenotypes in the mutant were caused by disruption of the AAH gene and are not due to an additional T-DNA insert or spontaneous mutation (O'Malley and Ecker 2010, Gase et al. 2011).

#### Expression of AtAAH during plant development

The expression pattern of *AtAAH* during plant development was analyzed by reverse transcription–quantitative PCR (RT–qPCR) in Col-0. In addition to its expression in pollen, the gene is expressed throughout plant development, but it is especially high in seedlings, roots and in siliques, 8 d after pollination (**Fig. 2A**).

Earlier, we identified AtAAH as a dormancy-up gene based on the higher expression in dormant compared with after-ripened seeds (Yazdanpanah et al. 2017). To investigate how the higher expression in dormant vs. after-ripened seeds matches the more dormant phenotype of the knock-out mutant, the expression pattern of AtAAH was analyzed in Cvi (Cape Verde Islands) seeds. This analysis shows that AtAAH expression in dry seeds is almost similar for dormant and non-dormant seeds, but it increased upon imbibition in both the micropylar and chalazal endosperm (ME), as well as the radicle and hypocotyl (RAD) of dormant seeds, but significantly less in non-dormant seeds (**Fig. 2B**).

### Exogenous potassium nitrate partly rescues the *Ataah* phenotype

To find out whether the increased seed dormancy of the mutant is a direct result of the lack of available nitrate, we applied exogenous potassium nitrate (KNO<sub>3</sub>) to the mother plant during seed development and to the seeds during seed imbibition. Nitrate assimilation of parent plants during seed maturation affects the accumulation of nitrate in seeds. Different KNO<sub>3</sub> regimes (N0, N5 and N20) resulted in corresponding (low in N0 and high in N20) nitrate levels in the mature seeds; these levels were similar for both the Ataah mutant and the wild type (Supplementary Fig. S2A). Treatment of the Ataah mutant with the highest nitrate level partly overcame the increased dormancy of the mutant compared with that of the wild-type Col-0 (Fig. 3A), but it could not prevent the seed abortion in the mutant. Similar results were obtained for the germination in salt and mannitol (Fig. 3B, C). The application of the highest nitrate concentration (20 mM) did completely abolish the difference between the wild type and mutant when germinated in mannitol (Fig. 3C). The germination of the mutant in ABA was not affected by the application of nitrate (Fig. 3D). Also the seed ABA levels did not differ either among the seeds that were grown in the different nitrate regimes or by mutant complementation (Supplementary Fig. S2B and C).

Freshly harvested (5 d after harvest) dry seeds that had developed under the three nitrate regimes were imbibed in three different nitrogen sources (nitrate or ammonium). For all genotypes, germination of seeds treated with KNO<sub>3</sub> was higher (P < 0.05) as compared with the germination in water (Fig. 4A). Thus, both wild-type and mutant seeds responded to exogenous KNO<sub>3</sub>, of which the mutant response was more obvious. The application of KNO<sub>3</sub> rescued the germination of the mutant, since the germination of the mutant with the addition of KNO<sub>3</sub> is higher than that of the wild type in water for all three nitrogen seed maturation regimes. Imbibition on  $NH_4NO_3$  and  $NH_4Cl$  did not affect the germination percentage (Fig. 4B, C), although NH<sub>4</sub> possibly had a toxic effect considering the fact that the non-germinating seeds turned dark (data not shown). Further it was investigated whether the effect of KNO<sub>3</sub> is the result of nitrate signaling or of nitrate assimilation. For nitrate assimilation, the conversion of NO<sub>3</sub> to NO<sub>2</sub> by nitrate reductase (NR) is required. Inhibiting NR by tungstate (Hilhorst and Karssen 1989) during seed germination did not inhibit the effect of KNO<sub>3</sub> on the partial complementation of the dormancy phenotype and therefore excludes an effect of nitrate as the nitrogen resource (Fig. 4D).

### Expression analysis of other genes in the purine pathway

To investigate whether the loss of function of ATAAH affected other genes in the purine pathway (AtALN, allantoinase; URE, urea hydrolase; UAH, ureidoglycolate amidohydrolase; and UGLYAH ureidoglycine aminohydrolase), RT–qPCR was performed on seeds of the Ataah mutant and its wild type (Col-0) matured in the different nitrate regimes. These analyses confirmed the absence of the relevant mRNA in the Ataah T-DNA insertion mutant (**Fig. 5A**). Further, only AtALN and URE displayed slightly, but significantly, higher expression in seeds of the Ataah mutant which had developed under N0, as compared





whoaded from https://academic.oup.com/pcp/article/63/9/1298/6647669 by Wageningen University and Research – Library user on 25 October 2022

Fig. 2 Expression pattern of ALLANTONATE AMIDOHYDROLASE (AtAAH) during plant development. (A) Mean relative expression levels of AtAAH measured by RT–qPCR in tissues of the Columbia accession leaf (L), flower (F), seedling (SE), root (R) and stem (ST) in siliques 3, 8 13 and 18 d after pollination (DAP), in dormant dry (D) and 6 h imbibed seeds (6hD), and across the germination time course in after-ripened dry (ARD) seeds and 6, 12, 24, 35, 48 and 73 h after imbibition. Expression values are normalized by the expression of two reference genes that are stably expressed in dry seeds: At4g12590 and At4g34270. (B) AtAAH expression patterns measured by qbasePLUS software in dormant and non-dormant (after-ripened) dry Cvi seeds and the micropylar and chalazal endosperm (ME) and radicle and hypocotyl (RAD) at 3, 7, 12 and 24 h after imbibition. Asterisks indicate significant differences between dormant and non-dormant in AtAAH expression (\*\*\*P < 0.001; \*\*P < 0.05).

with the other nitrate regimes (**Fig. 5B, C**). AtALN expression in the mutant was also significantly higher at N0 than that in Col-0 at the same concentration (**Fig. 5B**).

### Metabolic changes in the Ataah loss-of-function mutant

The levels of ureidoglycolate, allantoin and allantoate were determined in dormant dry seeds of Col-0 wild type, the *Ataah* mutant and two independent *Ataah* complementation lines. This analysis showed that mutant seeds had significantly higher

levels of allantoin and allantoate than the wild type (Fig. 6A). Moreover, complementation of the *Ataah* mutant with wildtype *AtAAH* reverted the mutant phenotype (Fig. 6A). There were no differences in ammonium contents between the Col-0 wild type, the *Ataah* mutant and two independent *Ataah* complementation lines (Fig. 6B).

GC-MS metabolite profiling of 24 h imbibed dormant seeds of Col-0 and the *Ataah* mutant revealed a higher urea abundance in the mutant (about 12 times more than Col-0). The contents of the amino acids serine, threonine, isoleucine and glycine were strongly reduced and there was a reduction in F. Yazdanpanah et al. | Role of allantoate amidohydrolase in seed germination





Fig. 3 Effect of different nitrate regimes during seed maturation on seed performance. Seed performance of both Ataah and Col-0 is presented. (A) Dormancy level (DSDS50), (B) germination in salt (130 mM NaCl), (C) germination in mannitol (-1 MPa), (D) germination in ABA (0.15  $\mu$ M) of seeds matured on plants exposed to different nitrate levels (N0, N5 and N20). Shown are averages of four biological replicates and their SE. Significant differences between Ataah and Col-0 are indicated by \*\*\* (P < 0.001) and different letters (Student *t*-tests; P < 0.05).

the organic acids malate, fumarate, citrate and succinate in the mutant as compared with the wild type (Fig. 6C).

#### Discussion

The final steps of purine degradation have long been the focus of research, especially because in tropical legumes these reactions are central to nitrogen supply under nitrogen-fixing conditions. Among the enzymes involved, a key role for AtAAH for recycling purine ring nitrogen has been demonstrated (Werner et al. 2008). In the present study we identified increased seed dormancy in seeds of the Ataah mutant. This phenotype is similar to that of a knock-out mutant of another gene in the same pathway, ALLANTOINASE (AtALN) (Piskurewicz et al. 2016). Loss-of function of AtALN has been reported to activate ABA metabolism in response to drought and osmotic stress in Ataln mutant seedlings (Watanabe et al. 2014b). ABA measurements in freshly harvested Ataah and Col-0 seeds produced under different nitrate regimes and in the mutant and complementation lines revealed that changes in ABA levels are not the cause of the increased dormancy of the Ataah mutant seeds (Supplementary Fig. S2BC).

AtAAH was found as a dormancy-up gene (Yazdanpanah et al. 2017), which suggests that its expression in dormant seeds is induced to overcome the block of germination by increasing internal nitrogen resources. In agreement with this, Takagi and coauthors reported that Ataln and Ataah mutants are relatively inefficient at using N for carbon assimilation and dry matter production. Applying exogenous nitrate could restore the growth of the Ataah mutant but not of the Ataln mutants (Takagi et al. 2016). To investigate whether indeed lack of nitrogen caused the increased dormancy in Ataah seed, we applied nitrogen to either the growth or the germination medium (Figs. 3 and 4). Application of high nitrate (20 mM) to the growth solution rescued, although not completely, the dormancy phenotype of the mutant. The higher exogenous nitrate content also clearly promoted the germination of after-ripened Ataah seeds under salt and osmotic stress, but did not affect germination in the presence of ABA. Ataah mutant seeds were hypersensitive to ABA, which suggests that the phenotype of the mutant is not directly related to ABA and ABA sensitivity. Moreover, among the various nitrogen compounds applied during the imbibition of freshly harvested seeds, only KNO<sub>3</sub> stimulated germination, and the highest concentration even completely rescued the dormancy phenotype of the mutant. In contrast to other species, ammonium nitrate did not promote germination in Arabidopsis seeds, but, on the contrary, probably exerted a toxic effect, as explained by the darkening of the seedlings. The fact that only nitrate affected the germination of the mutant and not the other nitrogen sources suggests an effect of nitrogen as a signaling molecule rather





Fig. 4 Effect of applying nitrogen-containing compounds during seed imbibition. Germination percentage of *Ataah* and Col-0 after applying (A) KNO<sub>3</sub> (10 mM), (B) NH<sub>4</sub>NO<sub>3</sub> (10 mM) and (C) NH<sub>4</sub>Cl (10 mM) during seed imbibition. Means of maximum germination percentage from four replicates are presented. Significant differences between *Ataah* and the control Col-0 are indicated (\*P < 0.05, \*\*P < 0.01 and \*\*\*P < 0.001). The experiments presented in (A–C) were all performed at the same time; the KNO<sub>3</sub>, NH<sub>4</sub>NO<sub>3</sub> and NH<sub>4</sub>Cl treatments are compared with the same water control. (D) Germination percentage of Col-0, *Ataah* and two independent *Ataah* complementation lines in the presence of the nitrate reductase inhibitor tungsten (Na<sub>2</sub>WO<sub>4</sub>), with and without addition of 10 mM KNO<sub>3</sub>. The experiment was performed 28 d after seed harvest when the *Ataah* seeds had not yet after-ripened. Means of maximum germination percentage from four replicates are presented. Differences are indicated by different letters (Student *t*-tests; P < 0.05).

than a nutritional role. This is confirmed by the fact that there is no difference in germination percentage when nitrate reductase is inhibited by the application of tungstate (Fig. 4D). The dormancy-breaking effect of nitrogen as a signal is well known (Alboresi et al. 2005) and has been reported to be similar to that of other germination stimulators, such as after-ripening, light and stratification (Finch-Savage et al. 2007). Apart from other signaling pathways, nitrate signaling may induce the expression of the ABA catabolic gene CYP707A2 (Ali-Rachedi et al. 2004, Alboresi et al. 2005, Matakiadis et al. 2009). Our investigations, as explained above, seem to exclude a role for ABA in the increased dormancy of the *Ataah* mutant.

Taken together, we hypothesize that the effect of KNO<sub>3</sub> is explained by the general effect that nitrate has on promoting germination and thus does not specifically rescue the dormancy phenotype of the *Ataah* mutant. Factors that might explain the AtAAH germination phenotype are discussed below. PCCP PLANT & CELL PHYSIOLOGY



Fig. 5 Relative expression of genes in the purine pathway related to allantoate degradation: AtAAH (A), AtALN (B), URE (C), UAH (D) and UGLYAH (E). Plant material consisted of seeds of Col and the *ataah* mutant matured on plants exposed to different nitrate levels. Means of relative expression level (reference genes At3g25800 and At4g34270) from four replicates are presented. Error bars represent the SEs, and different letters indicate statistically significant differences (Student *t*-tests; *P* < 0.05).



Fig. 6 (A) Metabolic profiles of seeds of Col-0 and the *Ataah* mutant. Allantoate, allantoate and ureidoglycolate concentrations in Col-0, *Ataah* and two independent *Ataah* complementation lines. (B) Ammonium content of Col-0, *Ataah* and two independent *Ataah* complementation lines. (C) Metabolite levels (mg DW<sup>-1</sup>) in 24 h imbibed dormant seeds of Col-0 and the *Ataah* mutant. Asterisks indicate significant differences relative to the respective wild type (\*\*\*P < 0.001; \*\*P < 0.01).



Wild-type plants exposed to low nitrogen conditions show reduced levels of allantoate, which supports the nutritional effect of nitrate and that ureides are a source of recycled nitrogen (Takagi et al. 2016). Ataah mutant seeds contain higher amounts of allantoin and allantoate due to the fact that these mutants lack a functional AtAAH which is required for the breakdown of these ureides (Todd and Polacco 2006). This is probably the reason for the lower abundance of the amino acids serine, threonine, isoleucine and glycine in Ataah seeds (Fig. 3). Consequently, the lack of these amino acids might inhibit the translation of transcripts that are necessary for germination. On the other hand, the reduced amino acid levels in the Ataah mutant seeds might be the result of, or the reason for, the higher sensitivity of the mutant seeds to salt and mannitol, since accumulation of compatible solutes such as amino acids is one of the mechanisms to deal with negative effects of salinity on plants (Giri 2011). Seeds of the Ataah mutant with increased dormancy also display a reduced energy metabolism and tricarboxylic acid (TCA) cycle activity, as compared with Col-0, and which can be concluded from the reduced amounts of malate, fumarate, citrate and succinate in the mutant compared with the wild type (Fig. 6B). It has been suggested that the TCA cycle is activated during seed germination (He et al. 2011). Thus, the low abundance of TCA cycle metabolites might be caused by the dormancy status of the mutant.

Moreover, there are several reports that consider allantoin and allantoate as reactive oxygen species (ROS) protectants (Takagi et al. 2016, Brychkova et al. 2008, Yesbergenova et al. 2005, Alamillo et al. 2010, Watanabe et al. 2014a, Irani and Todd 2016). These metabolites can act as scavengers of ROS and might contribute to stress protection during the maturation phase, dormancy and germination of seeds, as well as early growth of seedlings (Takagi et al. 2016). ROS have been shown to be important for overcoming seed dormancy (Oracz et al. 2007). Therefore, the overaccumulation of allantoin and allantoate in the *Ataah* mutant seeds may prevent the achievement of levels of ROS that are required to induce germination.

Ataah mutant seeds also accumulate urea, which might be explained by arginase activity. Arginine and ureides (such as allantoin and allantoate) represent the major potential sources of ammonium for incorporation into proteins as well as nucleic acids and a range of secondary metabolites (Trivedi 2006). Arginine breakdown is catalyzed by the mitochondrial arginase; this step involves arginine hydrolysis to ornithine and urea. In the *aah* mutant, allantoin and allantoate cannot be metabolized, the route via arginine might be activated and thus might result in urea accumulation.

In conclusion, the effect of AtAAH on seed germination is not completely revealed but the data presented here strongly suggest that AtAAH is required to support seed germination. When AtAAH is non-functional, this leads to an increase of seed dormancy. Preparing the seeds for seed germination already starts during seed maturation, which is also explained by the rather high expression of AtAAH during seed development and the up-regulation in dormant seeds (**Fig. 2A**). However, by far the highest transcript abundance was found in mature pollen (**Supplementary Fig. S1**). It is possible that the lack of this enzyme during early embryogenesis causes abortion of *Ataah* mutant seeds. Concerning the fact that siliques of mutated plants contain both normal and aborted seeds (**Fig. 1B**), it is more likely that abortion is a result of reduced fertilization efficiency of the pollen rather than being the consequence of a defect in embryogenesis of the mutant seeds.

#### **Materials and Methods**

#### **Plant material**

Arabidopsis thaliana wild accession Col-0 and the homozygous T-DNA insertion mutant Ataah (SALK\_112631; At4g20070) were used for these analyses. The homozygous T-DNA insertion mutant was isolated by a PCR-based reverse genetic screen for T-DNA insertions in the corresponding gene (Alonso et al. 2003).

The complementation lines with homozygous transgene insertions, the *Ataah* mutant complemented with 35S-AtAAH-HAStrep, were described before by Werner et al. (2008).

#### **Plant growth conditions**

Seeds were sown in Petri dishes on water-soaked filter paper followed by a 4 d cold treatment at 4°C, and transferred to a climate room at 22°C with continuous light for 3 d before planting. Germinated seedlings were grown on 4 × 4 cm Rockwool blocks in a growth chamber at 20°C/18°C (day/night) under a 16 h photoperiod of artificial light (150  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>) and 70% relative humidity. Plants were grown in a standard nutrient solution (**Supplementary Table S1**) and watered three times per week. For nitrate-treated plants, when flowering started, the KNO<sub>3</sub> regime of plants was changed to 0, 5 and 20 mM, resulting in corresponding (low in N0, standard in N5 and high in N20) nitrate levels in the mature seeds, with four biological replicates containing four plants per replicate for each condition.

#### **Germination assays**

Germination tests to monitor the release of seed dormancy were performed as described in Yazdanpanah et al. (2017) based on the germinator package developed by Joosen et al. (2010). In short, at several intervals during seed dry storage, until all seed batches had reached 100% germination, aliquots of 50-100 seeds of each genotype were evenly sown on a filter paper soaked with 0.7 ml of demineralized water in a 6 cm Petri dish. Petri dishes were placed in plastic trays containing a filter paper saturated with tap water and closed with transparent lids. Trays were incubated for 1 week in a climate chamber, illuminated by 38 W Philips TL84 fluorescent tubes at 8 W m<sup>-2</sup> in continuous light at 22°C. After that, the total number and the number of germinated seeds was scored and percentages calculated.

Seed dormancy levels (DSDS50: days of seed dry storage required to reach 50% germination) were quantified as described in Yazdanpanah et al. (2019). In brief, germination tests were performed weekly until all seed batches had germinated to >90%. A generalized linear model with a logit link was adapted to calculate DSDS50 as previously introduced (He et al. 2014).

Germination under stress conditions was performed on fully after-ripened seeds as described in Yazdanpanah et al. (2019). Stress conditions were: osmotic stress (-1 MPa mannitol; Sigma-Aldrich), salt stress (130 mM NaCl; Sigma-Aldrich) and ABA stress (0.15  $\mu$ M ABA; Duchefa Biochemie). ABA was dissolved in 10 mM MES buffer (Sigma-Aldrich) and the pH adjusted to 5.8. To determine seed longevity, an accelerated aging test was performed by incubating seeds



above a saturated ZnSO<sub>4</sub> solution ( $40^{\circ}$ C, 85% relative humidity) in a closed tank for 5 d (ISTA, 2022). After incubation, the seeds were taken out and germinated on demineralized water as described before.

The effect of exogenous nitrogen sources on germination was tested by applying  $KNO_3$  (10 mM),  $NH_4NO_3$  (10 mM) or  $NH_4CI$  (10 mM) to seeds that had been stored in dry conditions for 14 d since seed harvest.

Germination in the presence of the nitrate inhibitor was performed on seeds that had been stored in dry conditions for 28 d since seed harvest. As an inhibitor,  $1 \text{ mM Na}_2\text{WO}_4$  (sodium tungstate, Sigma 223336) was used. Germination was performed with and without 10 mM KNO<sub>3</sub>.

#### **Nitrate determinations**

Nitrate measurements were performed as described in He et al. (2014). In brief, 5 mg of seeds were boiled at 100°C for 15 min in 0.5 ml of 0.5 M HCl and 50 mg l<sup>-1</sup> *trans*-aconitate (internal standard). After centrifuging for 2 min at 13,000 rpm, 200  $\mu$ l of the supernatant was transferred to an HPLC vial.

HPLC analysis was performed on a Dionex ICS2500 system with an AS11-HC column and an AG11-HC guard column, and eluted with NaOH. The elution procedure was: 0–15 min linear gradient of 25–100 mM NaOH, then 15–20 min of 500 mM NaOH followed by 20–35 min of 5 mM NaOH. Flow rates were 1 ml min<sup>-1</sup> throughout the run. Contaminating anions in the eluents were removed using an anion trap column (ATC), installed between the pump and the sample injection valve. Anions were determined by conductivity detection. Background conductivity was decreased using an ASRS suppressor, with water as a counterflow. Peaks were identified and quantified using known external standards. The external standard of nitrate was NaNO<sub>3</sub>.

#### Ammonium measurement

Ammonium was measured by segmented flow analysis. A 50 mg aliquot of freshly harvested seeds was ground and 5 ml of demi water was added and heated to 80°C for 5 min. Clear supernatant was taken to a new tube. The colorimetric analysis was done on a Skalar San continuous flow analyzer connected to a 1050 autosampler using chemical method 155–324. (Skalar Inc., Buford, GA, USA).

### Gene expression analysis of AtAAH and other genes in the purine pathway

RNA was isolated using the Nucleospin RNA plant kit (Macherey-Nagel: 740949) according to the manufacturer's protocol with minor modifications as described in Yazdanpanah et al. (2019): 3–5 mg of freshly harvested seeds were used for the extraction. Lysis was performed using 315  $\mu$ l of buffer RAP, 35  $\mu$ l of Plant RNA Isolation Aid (Ambion: AM9690) and 3.5  $\mu$ l of  $\beta$ -mercaptoethanol (Sigma: M6250). Final RNA was eluted in 40  $\mu$ l of RNAse-free water. Quality and concentrations were measured by loading 2  $\mu$ l of RNA on an Xpose slide 40 (Bioke: TR230300) and measured on an Xpose (Bioke: TR112003). RNA integrity was checked on a 1% agarose gel. cDNA was synthesized from 750 ng of RNA using the iScript cDNA Synthesis Kit (Bio-Rad: 1708890) according to the manufacturer's protocol. cDNA was diluted 10 times with sterile milliQ water. For each sample 2.5  $\mu$ l of cDNA, 5  $\mu$ l of iQ SYBR green supermix (Bio-Rad: 1725125) and 0.5  $\mu$ l of primer mix (10  $\mu$ l of work solution) were added and supplemented with water to 10  $\mu$ l. RT–qPCR was performed on a CFX connect (Bio-Rad).

Sequences of the primers of the target genes are presented in **Supplementary Table S2**. Expression of the genes was normalized based on the expression of two reference genes that are stably expressed in dry seeds: At4g12590 and At4g34270 (Dekkers et al. 2012), as explained by Yazdanpanah et al. (2019). Expression was calculated by using qbasePLUS (Hellemans et al. 2007), which is commercially available software (Biogazelle, Ghent, Belgium, www.biogazelle.com).

#### Metabolite extraction and derivatization methods

The metabolite extraction was performed on 24 h imbibed dormant seeds of Col-0 and the Ataah mutant based on a previously described method (Roessner et al. 2000), with some modifications. For each genotype, metabolite extractions were performed on four biological replicates. For each sample, 5 mg of seeds pre-cooled in liquid nitrogen were homogenized in 2 ml tubes with two iron balls (2.5 mm) using a micro dismembrator (Mo Bio Laboratory). Then 233  $\mu$ l of methanol/chloroform (4:3) was added, together with 50  $\mu$ l of standard (0.13 mg ml<sup>-1</sup> ribitol), and mixed thoroughly. After 10 min of sonication, 66 µl of MO water was added to the mixture followed by vortexing and centrifugation (5 min, 15,000 rpm). The methanol phase was collected in a glass vial. Then 166  $\mu$ l of methanol/chloroform (1:1) was added to the remaining organic phase and kept on ice for 10 min. A 66  $\mu$ l aliquot of MQ water was added followed by vortexing and centrifugation (5 min, 15,000 rpm). Again the methanol phase was collected and mixed with the previously collected phase. A 60  $\mu$ l aliquot was dried overnight using a speedvac (room temperature, Savant SPD121). Dried samples were derivatized online as described by Lisec et al. (2006) using a Combi PAL autosampler (CTC Analytics). The derivatized samples were analyzed by a GC-TOF-MS system consisting of an Optic 3 highperformance injector (ATAS) and and Agilent 6890 gas chromatograph (Agilent Technologies) coupled to a Pegasus III time-of-flight (TOF) mass spectrometer (Leco Instruments). A 2  $\mu$ l aliguot of each sample was introduced to the injector. The details of the GC-TOF-MS method were as described by Carreno-Quintero et al. (2012) with some minor modifications. Detector voltage was set at 1,650 V.

#### ABA extraction and detection method

To measure ABA content, 10 mg of frozen dry seeds were ground in a 2 ml Eppendof tube using stainless steel beads. ABA was extracted and purified according to a protocol described by Zhou et al. (2003). ABA content was measured by injecting 10  $\mu$ l of extract into a Waters Xevo tandem quadruple mass spectrometer equipped with an electrospray ionization source and coupled to an Acquity UPLC BEH C18 column (100 mm) at 0.2 ml min<sup>-1</sup> with acetonitrile (ACN)/0.1% formic acid (FA), MQ/0.1% FA flow. ABA was quantified using a calibration curve with known amounts of ABA based on the ratio of the summed area of the multiple reaction monitoring transitions for ABA to those for [ $_2H_6$ ]ABA. Data acquisition was performed using MassLynx 4.1 software (Waters, USA).

#### **Supplementary Data**

Supplementary data are available at PCP online.

#### **Data Availability**

No new data were generated or analyzed in support of this research.

#### Funding

This work was supported by project 'How does nature regulate seed life span?' (with project number [12951] of the research program Applied and Engineering Sciences which is (partly) financed by the Dutch Research Council (NWO).

#### Acknowledgments

We thank Dr. Claus-Peter Witte for kindly providing seeds of complementation lines of the *Ataah* mutant. We would like to



thank Kristýna Floková and Diaan Jamar for excellent help with the ABA and metabolite analyses.

#### Disclosures

The authors have no conflicts of interest to declare.

#### References

- Alamillo, J.M., Díaz-Leal, J.L., SÁnchez-Moran, M.V., Pineda, M., (2010) Molecular analysis of ureide accumulation under drought stress in Phaseolus vulgaris L. *Plant Cell Environ*. 33: 1828–1837.
- Alboresi, A., Gestin, C., Leydecker, M.T., Bedu, M., Meyer, C. and Truong, H.N. (2005) Nitrate, a signal relieving seed dormancy in Arabidopsis. *Plant Cell Environ*. 28: 500–512.
- Ali-Rachedi, S., Bouinot, D., Wagner, M.H., Bonnet, M., Sotta, B., Grappin, P., et al. (2004) Changes in endogenous abscisic acid levels during dormancy release and maintenance of mature seeds: studies with the Cape Verde Islands ecotype, the dormant model of *Arabidopsis thaliana*. *Planta* 219: 479–488.
- Alonso, J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shinn, P., et al. (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* 301: 653–657.
- Arc, E., Sechet, J., Corbineau, F., Rajjou, L. and Marion-Poll, A. (2013) ABA crosstalk with ethylene and nitric oxide in seed dormancy and germination. *Front. Plant Sci.* 4: 63.
- Bentsink, L. and Koornneef, M. (2008) Seed dormancy and germination. *Arabidopsis Book* 6: e0119.
- Bewley, J.D. (1997) Seed germination and dormancy. *Plant Cell* 9: 1055-1066.
- Bewley, J.D.Bradford, K.J., Hilhorst, H.W.M. and Nonogaki, H. (2013) Environmental regulation of dormancy and germination. *In* Seeds. pp. 299–339. New York: Springer.
- Brychkova, G., Alikulov, Z., Fluhr, R. and Sagi, M. (2008) A critical role for ureides in dark and senescence-induced purine remobilization is unmasked in the Atxdh1 Arabidopsis mutant. *The Plant Journal*. 54: 496–509.
- Carreno-Quintero, N., Acharjee, A., Maliepaard, C., Bachem, C.W., Mumm, R., Bouwmeester, H., et al. (2012) Untargeted metabolic quantitative trait loci analyses reveal a relationship between primary metabolism and potato tuber quality. *Plant Physiol.* 158: 1306–1318.
- Dekkers, B.J., Willems, L., Bassel, G.W., van Bolderen-veldkamp, R.M., Ligterink, W., Hilhorst, H.W., et al. (2012) Identification of reference genes for RT-qPCR expression analysis in Arabidopsis and tomato seeds. *Plant Cell Physiol*. 53: 28–37.
- Diaz, C., Lemaître, T., Christ, A., Azzopardi, M., Kato, Y., Sato, F., et al. (2008) Nitrogen recycling and remobilization are differentially controlled by leaf senescence and development stage in Arabidopsis under low nitrogen nutrition. *Plant Physiol.* 147: 1437–1449.
- Finch-Savage, W.E., Cadman, C.S., Toorop, P.E., Lynn, J.R. and Hilhorst, H.W. (2007) Seed dormancy release in Arabidopsis Cvi by dry after-ripening, low temperature, nitrate and light shows common quantitative patterns of gene expression directed by environmentally specific sensing. *Plant J.* 51: 60–78.
- Finch-Savage, W.E. and Leubner-Metzger, G. (2006) Seed dormancy and the control of germination. *New Phytol.* 171: 501–523.
- Gase, K., Weinhold, A., Bozorov, T., Schuck, S. and Baldwin, I.T. (2011) Efficient screening of transgenic plant lines for ecological research. *Mol. Ecol. Resour.* 11: 890–902.
- Giri, J. (2011) Glycinebetaine and abiotic stress tolerance in plants. *Plant Signal. Behav.* 6: 1746–1751.

- Hanks, J.F., Tolbert, N. and Schubert, K.R. (1981) Localization of enzymes of ureide biosynthesis in peroxisomes and microsomes of nodules. *Plant Physiol.* 68: 65–69.
- He, D., Han, C. and Yang, P. (2011) Gene expression profile changes in germinating rice. J. Integr. Plant Biol. 53: 835-844.
- He, H., de Souza Vidigal, D., Snoek, L.B., Schnabel, S., Nijveen, H., Hilhorst, H., et al. (2014) Interaction between parental environment and genotype affects plant and seed performance in Arabidopsis. J. Exp. Bot. 65: 6603–6615.
- He, H., Willems, L.A., Batushansky, A., Fait, A., Hanson, J., Nijveen, H., et al. (2016) Effects of parental temperature and nitrate on seed performance are reflected by partly overlapping genetic and metabolic pathways. *Plant Cell Physiol.* 57: 473–487.
- Hellemans, J., Mortier, G., De Paepe, A., Speleman, F. and Vandesompele, J. (2007) qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol.* 8: R19.
- Hilhorst, H.W.M. (1995) A critical update on seed dormancy. I. Primary dormancy. Seed Sci. Res. 5: 61-73.
- Hilhorst, H.W.M. and Karssen, C.M. (1989) Nitrate reductase independent stimulation of seed germination in Sisymbrium officinale L. (hedge mustard) by light and nitrate. Ann. Bot. 63: 131–137.
- Irani, S. and Todd, C.D. (2016) Ureide metabolism under abiotic stress in Arabidopsis thaliana. J Plant Physiol. 199: 87–95.
- ISTA (2022) The international rules for seed testing. Bassersdorf: International Seed Testing Association.
- Joosen, R.V., Kodde, J., Willems, L.A., Ligterink, W., van der Plas, L.H. and Hilhorst, H.W.M. (2010) GERMINATOR: a software package for highthroughput scoring and curve fitting of Arabidopsis seed germination. *Plant J.* 62: 148–159.
- Lisec, J., Schauer, N., Kopka, J., Willmitzer, L. and Fernie, A.R. (2006) Gas chromatography mass spectrometry-based metabolite profiling in plants. *Nat. Protoc.* 1: 387–396.
- Maathuis, FJ. (2009) Physiological functions of mineral macronutrients. *Curr. Opin. Plant Biol.* 12: 250–258.
- Malagoli, P., Laine, P., Rossato, L. and Ourry, A. (2005) Dynamics of nitrogen uptake and mobilization in field-grown winter oilseed rape (*Brassica napus*) from stem extension to harvest. II. An <sup>15</sup>N-labelling-based simulation model of N partitioning between vegetative and reproductive tissues. *Ann. Bot.* 95: 1187–1198.
- Matakiadis, T., Alboresi, A., Jikumaru, Y., Tatematsu, K., Pichon, O., Renou, J.P., et al. (2009) The Arabidopsis abscisic acid catabolic gene CYP707A2 plays a key role in nitrate control of seed dormancy. *Plant Physiol.* 149: 949–960.
- O'Malley, R.C. and Ecker, J.R. (2010) Linking genotype to phenotype using the Arabidopsis unimutant collection. *Plant J.* 61: 928–940.
- Oracz, K., El-Maarouf Bouteau, H., Farrant, J.M., Cooper, K., Belghazi, M., Job, C., et al. (2007) ROS production and protein oxidation as a novel mechanism for seed dormancy alleviation. *Plant J.* 50: 452–465.
- Piskurewicz, U., Iwasaki, M., Susaki, D., Megies, C., Kinoshita, T. and Lopez-Molina, L. (2016) Dormancy-specific imprinting underlies maternal inheritance of seed dormancy in *Arabidopsis thaliana*. *eLife* 5: e19573.
- Ramazzina, I. (2006) Completing the uric acid degradation pathway through phylogenetic comparison of whole genomes. *Nat. Chem. Biol.* 2: 144.
- Roessner, U., Wagner, C., Kopka, J., Trethewey, R.N. and Willmitzer, L. (2000) Simultaneous analysis of metabolites in potato tuber by gas chromatography-mass spectrometry. *Plant J.* 23: 131-142.
- Smil, V. (2000) Cycles of Life. Scientific American Library, New York.
- Soppe, W.J.J. and Bentsink, L. (2020) Seed dormancy back on track; its definition and regulation by DOG1. *New Phytol.* 228: 816–819.
- Takagi, H., Ishiga, Y., Watanabe, S., Konishi, T., Egusa, M., Akiyoshi, N., et al. (2016) Allantoin, a stress-related purine metabolite, can activate



jasmonate signaling in a MYC2-regulated and abscisic acid-dependent manner. J. Exp. Bot. 67: 2519–2532.

- Tipton, P.A. (2006) Urate to allantoin, specifically (S)-allantoin. *Nat. Chem. Biol.* 2: 124–125.
- Todd, C.D. and Polacco, J.C. (2006) AtAAH encodes a protein with allantoate amidohydrolase activity from *Arabidopsis thaliana*. *Planta* 223: 1108–1113.
- Trivedi, P. (2006) Advances in Plant Physiology. IK International Pvt Ltd., Delhi.
- Vogels, G.D. and Van der Drift, C. (1976) Degradation of purines and pyrimidines by microorganisms. *Bacteriol. Rev.* 40: 403–468.
- Watanabe, S., Kounosu, Y., Shimada, H. and Sakamoto, A. (2014a) Arabidopsis xanthine dehydrogenase mutants defective in purine degradation show a compromised protective response to drought and oxidative stress. *Plant Biotechnol.* 31: 173–178.
- Watanabe, S., Matsumoto, M., Hakomori, Y., Takagi, H., Shimada, H. and Sakamoto, A. (2014b) The purine metabolite allantoin enhances abiotic stress tolerance through synergistic activation of abscisic acid metabolism. *Plant Cell Environ*. 37: 1022–1036.

- Werner, A.K., Sparkes, I.A., Romeis, T. and Witte, C.-P. (2008) Identification, biochemical characterization, and subcellular localization of allantoate amidohydrolases from Arabidopsis and soybean. *Plant Physiol.* 146: 418–430.
- Yazdanpanah, F., Hanson, J., Hilhorst, H.W.M. and Bentsink, L. (2017) Differentially expressed genes during the imbibition of dormant and after-ripened seeds—a reverse genetics approach. *BMC Plant Biol.* 17: 151.
- Yazdanpanah, F., Maurino, V.G., Mettler-Altmann, T., Buijs, G., Bailly, M.N., Karimi Jashni, M., et al. (2019) NADP-MALIC ENZYME 1 affects germination after seed storage in *Arabidopsis thaliana*. *Plant Cell Physiol*. 60: 318–328.
- Yesbergenova, Z., Yang, G., Oron, E., Soffer, D., Fluhr, R. and Sagi, M. (2005) The plant Mo-hydroxylases aldehyde oxidase and xanthine dehydrogenase have distinct reactive oxygen species signatures and are induced by drought and abscisic acid. *The Plant Journal* 42: 862–876.
- Zhou, R., Squires, T.M., Ambrose, S.J., Abrams, S.R., Ross, A.R. and Cutler, A.J. (2003) Rapid extraction of abscisic acid and its metabolites for liquid chromatography-tandem mass spectrometry. J. Chromatogr. A 1010: 75–85.