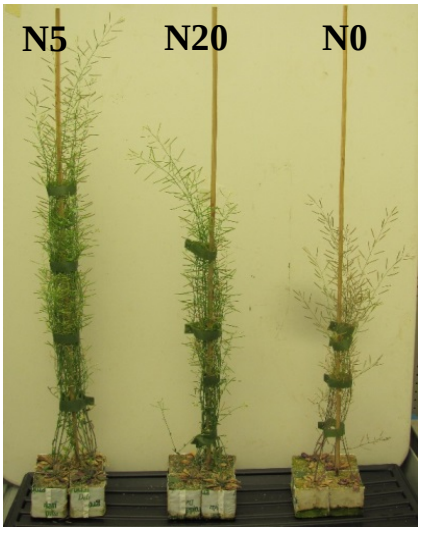
Wageningen University

Laboratory of Plant Physiology

Wageningen Seed Lab

MSc Thesis (PPH-80424)

Functional analyses of Allantoate Amidohydrolase



Rong Wei

Wageningen
May 2014 – August 2014

Wageningen University

Laboratory of Plant Physiology

Wageningen Seed Lab

MSc Thesis (PPH-80424)

Functional analyses of Allantoate Amidohydrolase

Rong Wei

(Registration No: 910406936010)

Supervisors:

Dr. ing. Leónie Bentsink

Farzaneh Yazdanpanah

Wageningen
May 2014 – August 2014

Preface

My deepest gratitude goes first and foremost to my supervisors, Farzaneh Yazdanpanah and dr. ing. Leónie Bentsink, for their patience, guidance and unconditional support. From them, I learnt how to perform real research and how to write in a scientific way. Their academic attitude and enthusiasm for research make a strong impression on me. I would never be able to accomplish my minor master thesis without their help. I would like to thank them very much for their support and understanding for the last four months.

I am feeling very lucky to do the minor thesis here in Seed lab. I appreciate the working atmosphere here as everyone share with each other, help each other and care about each other. I would like to thank all the staffs of Seed lab involved in this project: to post doc Bas Dekkers, technicians Kerstin Guhl, Leo Willems and Juriaan Rienstra. Also, I want to thank Di Lei for her help during the molecular experiments.

Most important, I would like to express thanks to my family. When I meet difficulties, they always stand by me and give me support and encouragement. Thank you all my friends, as we shared lots of precious moments.

It is my honour to study in Wageningen University and to do my minor master thesis in the Wageningen Seed Lab. All the experiences here will stay with me in my whole life.

Abstract

Allantoate amidohydrolase (AAH) is an enzyme in the final stage of purine catabolism, where uric acid catabolizes to produce nitrogen compounds in plants and some bacteria. Previous research showed that knock-out mutant Arabidopsis seed of this gene is more dormant and more sensitive to salt and mannitol. Arabidopsis seeds of this *aah* mutant and wild type harvested from plants treated with three levels of nitrate solution during seed maturation were used in our research to investigate the effect of nitrogen on seed dormancy. Gene expression was also performed to have a wider view of how seed responses to low nitrate condition. The result showed that mutant seed matured in low nitrate had higher dormancy level, and lower amount of ABA contents compare to wild type seeds.

High concentration of nitrate applied on parent plants had no effect on mutant seed dormancy, but enhanced seeds tolerance to stress conditions. Feeding with KNO_3 released the dormancy of mutant seeds especially of that matured in low nitrate condition, while the application of NH_4NO_3 and NH_4Cl were observe to have no positive effect on seed germination. Urea, malic acid and fumaric acid supplement had positive effects on dormancy release of mutant seeds obtained from intermediate nitrate level irrigation, and fumaric acid was observed to decrease germination of wild type seeds. Two genes, *ALN* and *URE* expressed to a higher level in mutant seeds matured with low nitrate, which might due to the activation of other nitrogen (ammonia) producing pathways to compensate the lacking of nitrogen. Seed nitrogen content determination, as well as feeding amino acids on seeds matured in low nitrate condition, is suggested to be executed in further research to have a more comprehensive view of AAH function.

Keywords: Allantoate amidohydrolase (AAH), seed dormancy, nitrogen, nitrate, ammonia

Table of Contents

1. Introduction

1.1 General introduction

Seed dormancy was defined by Bewley (1997) as the failure of an intact viable seed to complete germination under favourable conditions caused by physical, mechanical, or chemical inhibition. Environment factors including light (i.e. light quality, photoperiod), temperature, water, nutrients, the duration of seed storage, as well as mother plant growth conditions, would influence the level seed dormancy (Bewley, 1997; Bewley *et al.*, 2013). The relationship between seed dormancy and germination is shown in Figure 1.1.

Plants grown under nitrogen-limiting conditions possess means to optimize the use of available nitrogen by redistributing it from source to sink tissues (Masclaux-Daubresse *et al.*, 2010). Plant possesses enzymes that can concert nucleobase nitrogen into amino acid nitrogen, then usually used for long-distance nitrogen transport. In *Arabidopsis thaliana*, this conversion involved the pyrimidine bases, uracil and thymine catabolized in three enzymatic steps, followed by ammonia re-assimilation as well as transamination reactions (Zrenner *et al.*, 2009). The catabolism of the purine ring from the nucleotides AMP and GMP converges on xanthine, involving eight enzymatic reactions in the degradation pathway suggested in *Arabidopsis* (Werner and Witte, 2011). The catabolism of the purine ring system is shown in Appendix I.

Allantoate amidohydrolase (AAH) is an enzyme in the uric acid catabolic pathway. 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 (Zrenner *et al.*, 2006). The further catabolism of ureides follows one of two pathways (Figure 1.2). The degradation of allantoate is catalysed by either allantoicase or AAH. Allantoicase produces urea and ureidoglycolate, both of which require further catabolism for full nitrogen assimilation, and AAH results in ureidoglycine and ammonia (French and Ealick, 2010). AAH has been identified as a gene that is up-regulated in 24h imbibed dormant seeds. Arabidopsis seeds of the gene knock-out (KO) mutant are more dormant and sensitive to salt and mannitol, which was found in preliminary experiments. The similar pattern between salt and mannitol indicates that the inhibition of the germination is likely an osmotic effect. Siliques of the homozygous KO line contains some aborted seeds (Figure 1.3-A), since this gene highly express in pollen (Figure 1.3-B) it may play a role during fertilisation.

It has been well known that conditions favoring nitrate accumulation in mother plants and in seeds lead to lower seed dormancy level (Alboresi *et al.*, 2005). A key gene in purine pathway to produce ammonia, the knock-out of *aah* may increase the dormancy level in Arabidopsis seeds. Metabolomic analysis on dormant seeds of this T-DNA line (imbibed) showed a high abundance of urea (about 12 times more than Col) and reduced abundance of amino acids serine, threonine, isoleucine and glycine.

Probably the nitrogen requirement of mutant seeds activates the purine pathway and since this enzyme (AAH) is missing, allantoate is converted to ureidoglycolate which leads to the producing of urea (known as the urea producing pathway) instead of the conversion to uredoglycine which form ammonia as a side product (known as ammonia producing pathway), and it end up with high accumulation of urea in seeds. Preliminary experiments showed that the TCA cycle and energy metabolism produced less abundant amino acids such as citric acid, malic acid, succinic acid and fumaric acid in the mutant compare to wild type (Figure 1.4). This suggested that lacking of *aah* in purine pathway might affect the biosynthesis of amino acids related to xanthine metabolism.

Metabolite analysis of Arabidopsis seeds developed in different nitrate regimes showed that allantoin and urea content are reduced in low nitrate conditions compared to seeds developed in control conditions (Hanzi He, unpublished), which could be a reason of seed dormancy caused by parent plants. Thus the effect of exogenous nitrogen compounds provided to seeds on dormancy will be interesting to be investigated.

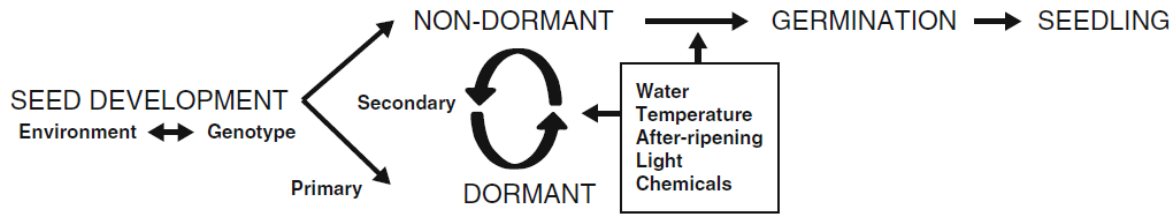


Figure 1.1 Relationships between seed dormancy and germination. Seeds can be dormant (primary dormancy) or non-dormant at the end of seed development depending upon both genotype and maternal environment. Dormancy can be alleviated by various environmental factors. Alternatively, non-dormant seeds can be induced into dormancy (secondary dormancy) by some of the same factors. Seeds may cycle seasonally between the dormant and non-dormant. Non-dormant seeds can progress to germination, again influenced by some of the same environmental factors (Bewley *et al*., 2013).

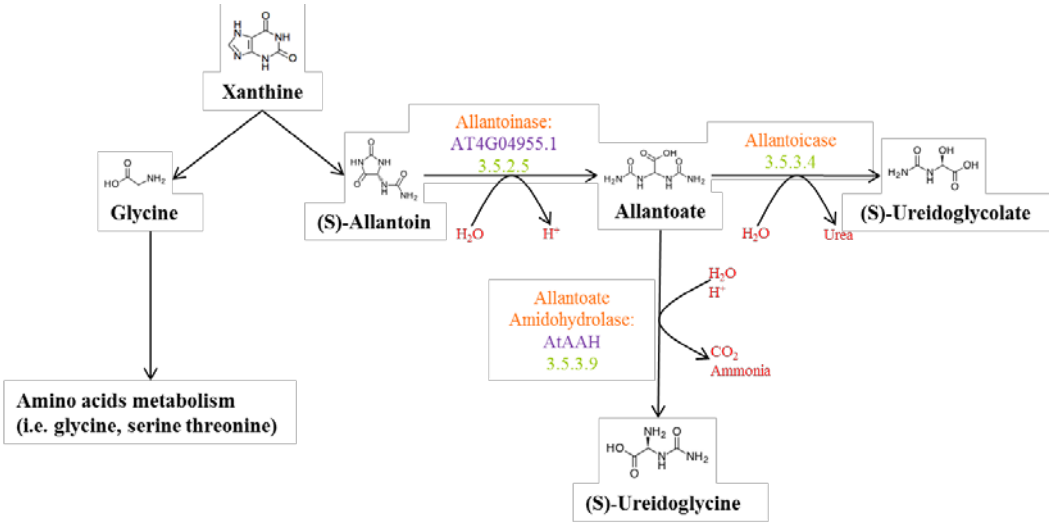


Figure 1.2 Simplified catabolism pathway of the purine ring system beginning with xanthine (KEGG Purine metabolism – Reference pathway).

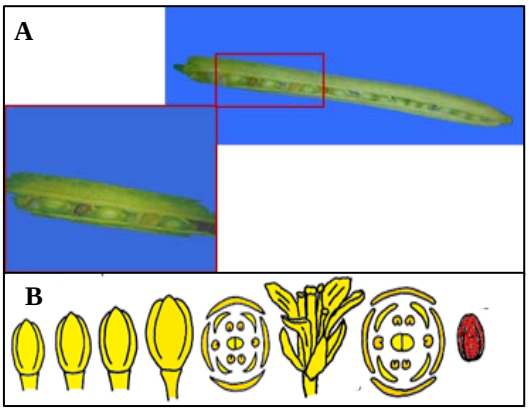


Figure 1.3 Seed abortion in siliques of *Ataah* mutant (A) and high expression of this gene in schematic presentation of an Arabidopsis flower bud (B). Above the full silique, red rectangular indicated zoom in to part of siliques where seed abortion is found in subfigure A. In subfigure B, red colour indicates higher gene expression and yellow indicates a lower expression level.

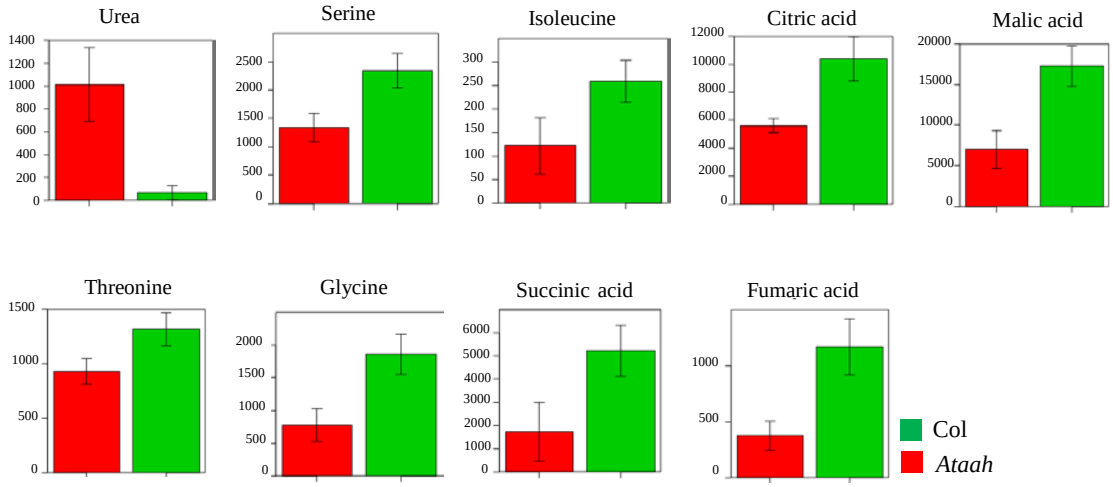


Figure 1.4 Metabolites with significantly different amounts in the *Ataah* mutant compared to wild type (Col) seeds.

1.2 Aims

1. To identify the effect of nitrate applied to mother plant during seed maturation stage on the seed dormancy levels of both mutant and wild type (WT).
2. To determine whether providing nitrate on mother plants during seed maturation will enhance stress tolerance ability of mutant seeds compare to WT.
3. To investigate the effect of external nitrogen supplement during germination on seed dormancy of both mutant and WT seeds matured in different concentrations of nitrate solution.
4. To investigate how seed responses to ammonia lacking when *AAH* is deleted.

1.3 Hypothesis

1. The dormancy of *Ataah* mutant can be relieved by maturation on mother plants grow at high nitrate level
2. The sensitivity to mannitol, salt and ABA of *Ataah* mutant can be reduced by maturation on mother plants grow at high nitrate level.
3. Feeding nitrogen components during germination could release dormancy of mutant seeds.
4. Plants compensate the low nitrate condition by activating the purine pathway.

2 Material and Methods

2.1 Plant materials

The *Arabidopsis thaliana* seeds Columbia (Col-0) was used as WT, and mutant seeds was in the Col-0 background with T-DNA line defective in allantoate amidohydrolase (SALK_112631; At4g20070).

2.2 Growth conditions

Seeds were sown in petri dishes on water soaked filter paper followed by a 4-day cold treatment at 4°C and transferred to a climate room at 22°C with continuous light for 3 days before planting. Germinated seedlings were grown on 4 x 4 cm Rockwool blocks in a climate chamber at Radix Kilma (Bornsesteeg 48, Building 109, Wageningen, the Netherlands) maintained at 20°C/18°C (day/night) under a 16-h photoperiod of artificial light (150 μmol m^{-2} s^{-1}) and 70% relative humidity. Plants were grown in a standard nutrient solution (Appendix II) and irrigated three times per week. Upon the start of flowering, the plants were divided to three trays, with four biological replicates containing four plants per replicate in one tray, and treated with nutrient solutions of different nitrogen concentrations. The original nutrient solutions were made into three nitrate levels: 0, 10 and 40 mM, and water was added to dilute the concentration of half when applying solutions to plants (N0, N5, N20).

2.3 Seed phenotyping

Seeds of all treatments were harvested when the siliques had turned yellow and dry on the plant (Huang *et al.*, 2010) and pictures of plants were taken before to determine the plant phenotype differences. Seeds were harvested as a bulk from four plants.

2.3.1 Seed dormancy determination

Germination experiments were performed as described previously (Joosen *et al.*, 2010). In brief, two layers of blue germination paper were equilibrated with 49 ml demineralized water in plastic trays (15 x 21 cm). Six samples of approximately 50 to 150 seeds were spread on wetted papers using a mask to ensure accurate spacing. Piled up trays were wrapped in a closed transparent plastic bag. The experiment was carried out in a 22°C incubator under continuous light (143 μmol m^{-2} s^{-1}). Pictures were taken once a day for a period of 6 days using a Nikon D80 camera fixed to a repro stand with a 60mm macro objective. The camera was connected to a computer with Nikon Camera Control Pro software version 2.0.

Germination was scored using the Germinator package (Joosen *et al.*, 2010). To quantify seed dormancy (DSDS50: days of seed dry storage required to reach 50% germination), germination tests were performed weekly until all seed batches had germinated for more than 90%. A generalized linear model with a logit link as described by Hurtado *et al.* (2012) was adapted to calculate DSDS50.

2.3.2 Stress germination

Germination under stress conditions was performed on fully after-ripened seeds. Stress conditions were: osmotic stress (-0.8 MPa mannitol; Sigma-Aldrich), salt stress (110 mM NaCl; Sigma-Aldrich), ABA stress (0.12 µM ABA; Duchefa Biochemie). ABA was dissolved in 10 mM MES buffer (Sigma-Aldrich) and the pH adjusted to 5.8.

2.4 Feeding experiment

Feeding experiment was performed on dormant seeds. Chemicals used for feeding experiments were: KNO_3 (10mM; Sigma-Aldrich), NH_4NO_3 (5, 10, 20, 25 mM, Sigma-Aldrich), NH_4Cl (5, 20 mM, Sigma-Aldrich), NH_4OH ($NH_3 \cdot H_2O$; 5, 10 mM, Sigma-Aldrich) and amino acids (urea, serine, isoleucine, citric acid, malic acid, threonine, glycine, succinic acid, fumatic acid; 10 mM, Sigma-Aldrich)

2.5 Germination parameters

Maximum germination (Gmax) values were extracted from the germination assay using the Germinator package (Joosen *et al.*, 2010). Gmax is the final germination percentage at the end of the germination assay.

2.6 ABA determination

To measure ABA content, 10 mg of frozen dry seeds were grinded 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 µl into 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 with ACN/0.1FA, MQ/0.1FA flow. ABA was quantified using a calibration curve with known amount of ABA based on the ratio of the summed area of the MRM transitions for ABA to those for [2H6]-ABA. Data acquisition was performed using MassLynx 4.1 software (Waters, USA).

2.7 Gene expression analysis

For RNA isolation and qRT-PCR analysis, frozen seeds were ground in a 2ml eppendof tube using stainless steel beads. Total RNA was extracted according to the hot borate protocol from Maia *et al.* (2011). RNA samples were DNase treated, checked for the absence of DNA by PCR and used for cDNA synthesis.

RNA was reverse transcribed using the iScriptTM cDNA synthesis kit (Bio-Rad). In total 1 µg of total RNA was reverse transcribed according to the protocol. cDNA samples were diluted with sterile milliQ water. For each qPCR, 1 µl of sample, 12.5 µl of iQ SYBR Green Supermix (Bio-Rad) and 1 µl of primer (0.5 µl forward primer + 0.5 µl reverse primer from 10 mM working solution) was added and supplemented with water to a final volume 25 ml. The RT–qPCRs were run on a MyiQ (Bio-Rad). The qPCR program run consisted of a first step at 95°C for 3 min and afterwards 40 cycles alternating

between 15 s at 95°C and 1 min at 60°C. Reference gene used for qPCR was At3g25800. Primers designed for qPCR are listed below:

AAH (GenBank accession: AT4G20070):
Forward primer-5'-CATTGAACGAAAGCACGATG-3'
Reverse primer-5'-TCCTGCTCCACTCATTAGCA-3'.
ALN (AT4G04955):
Forward primer-5'-AGCAAAAGGCAAAGGAGACA-3',
Reverse primer-5'-CTGGAATCTCTTCGGCTGAG-3'.
UGLYAH (AT4G17050):
Forward primer-5'-ATCGCTTGGGTGATAACTGG-3',
Reverse primer-5'-CAGCATACCATTGAGGGACA-3'.
URE (AT1G67550):
Forward primer-5'-GCATTTCGTGGGAGAACAAT-3',
Reverse primer-5'-TGGTTGATGACGGGAGTACA-3'.
ATAAH2/UAH (AT5G43600):
Forward primer-5'-ATCTGCCTGCTGTAGCCACT-3',
Reverse primer-5'-AAACACCGACAACACCATCA-3'.

2.9 Data analysis

Data analysis on seed dormancy (days of dry storage to reach 50% germination, DSDS50) was done in the statistical programming environment R 2.14.1. One way ANOVA with $P \leq 0.05$ using the statistical analysis software GENSTAT 16th edition for Windows was performed for the experiments. Multiple comparisons among means of different treatments were performed using the Fisher's protected LSD test.

3 Result

To investigate the effect of parental environment on the capacity of seed germination, the *Arabidopsis thaliana* plants of *aah* mutant and Columbia (Col-0) from flowering onwards were grown in high, control and low nitrate solutions (0, 5, 20 mM). Our results show that low nitrate supplement during seed maturation increased dormancy of the mutant seeds and higher nitrate had no dormancy decreasing effect. For Col plants grown in three nitrate levels, the dormancy levels were similar, but significantly lower than mutant (Figure 3.1).

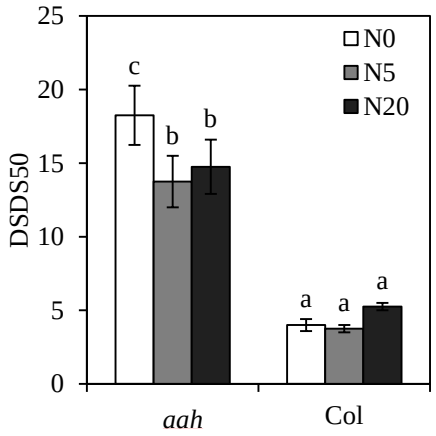


Figure 3.1 Dormancy level (DSDS50) of *aah* mutant and Col seeds matured in different nitrate levels. Means of four replicates are presented. Error bars represent standard errors, and different letters indicate statistical significant differences (Student t-tests; $P < 0.05$).

After-ripened seeds (almost 100% germination in water) were exposed to salt (Figure 3.2-A), mannitol (Figure 3.2-B) and ABA (Figure 3.2-C) to investigate the effect of stress conditions on seeds germination of the mutant compare to wild type. Mutant seeds from all nitrate treatments showed more sensitivity to salt and mannitol, but within the mutant, the ones from high nitrate treatment could tolerate the saline condition better than others. However in osmotic stress condition, mutant seeds matured in high nitrate condition did not decrease seed dormancy. For both mutant and wild type, seeds matured in control nitrate condition were better germinated than other treatments. Overall, osmotic stress reduced the germination rate in mutant compare to wild type seeds regardless of nitrate solution applied on parent plants. When applying ABA on seeds during germination, it promotes germination of mutant seeds compare to wild type seeds in all N treatments. Meanwhile, mutant seeds matured in high nitrate level had highest germination percentage.

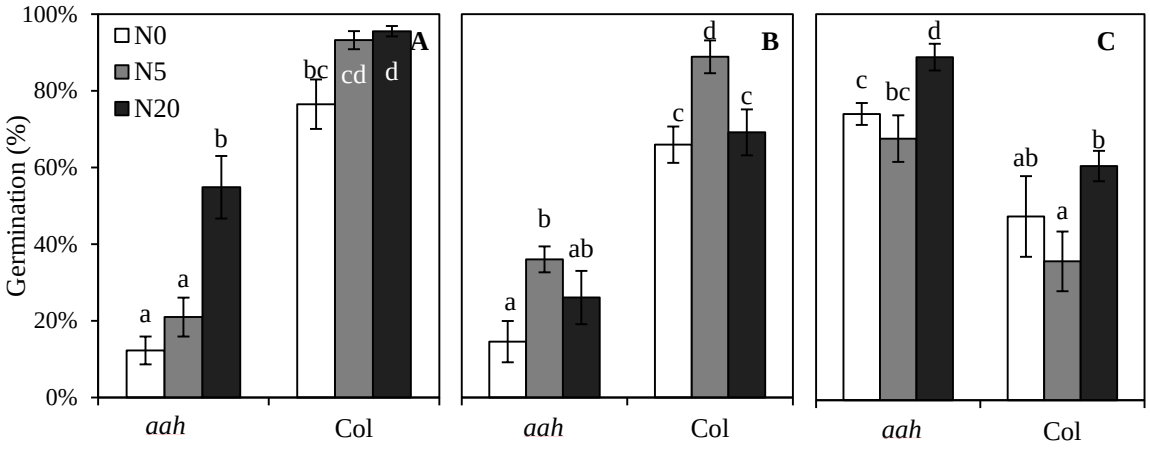


Figure 3.2 Salt (110 mM NaCl, A), mannitol (-0.8 MPa, B) and ABA (0.12 mM, C) stress germination of *aah* mutant and Col matured in different nitrate levels. Means of maximum germination percentage from four replicates are presented. Error bars represent standard errors, and different letters indicate statistical significant differences (Student t-tests; $P < 0.05$)

Nitrogen compounds in different forms (nitrate, ammonium) were fed to dormant seeds of mutant and WT to investigate the effect of external applied nitrogen compounds on releasing seed dormancy (Figure 3.3). KNO_3 increased the germination rate of the mutant especially for the seeds harvested from low nitrate condition, while for wild type seeds matured in all N levels KNO_3 did not make a difference in germination compare to non-treated ones(Figure 3.3-A). On the contrary, when seeds were supplied with NH_4NO_3 or NH_4Cl, only mutant seeds matured in low nitrate condition showed higher germination. Moreover, the seed germination percentage of wild type seed matured in 5 and 20 mM nitrate solution were largely reduced comparing to the ones without nitrogen feeding (Figure 3.3-B,C). When NH_4OH was fed on seeds, all seeds were observed turning brown one day after incubation.

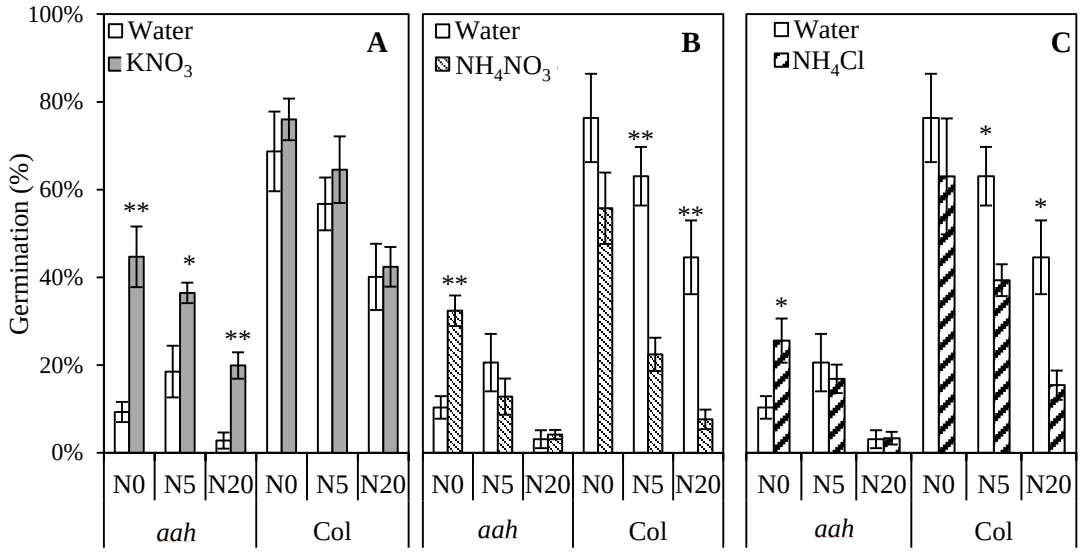


Figure 3.3 Germination percentage of seeds applied with KNO_3 (10 mM, A), NH_4NO_3 (20 mM, B) and NH_4Cl (20 mM, C) during germination of *aah* mutant and WT seeds matured in different nitrate levels. Means of maximum germination percentage from four replicates are presented. Error bars represent standard errors. * indicates the P value < 0.05 and ** indicates $P < 0.01$ (Student t-tests).

Mutant and WT seeds matured in 5mM nitrate solution were used to germinate in the presence of amino acids (Figure 3.4) to investigate their effect on releasing dormancy. Overall, wild type seeds in amino acids (and water) had higher germination percentage compare to mutant seeds, except fumaric acid where the germination of WT was largely inhibited. Figure 3.4 shows that when applying urea, malic acid and fumaric acid during germination on mutant seeds, the seed dormancy was released compare to WT seeds germinated in water.

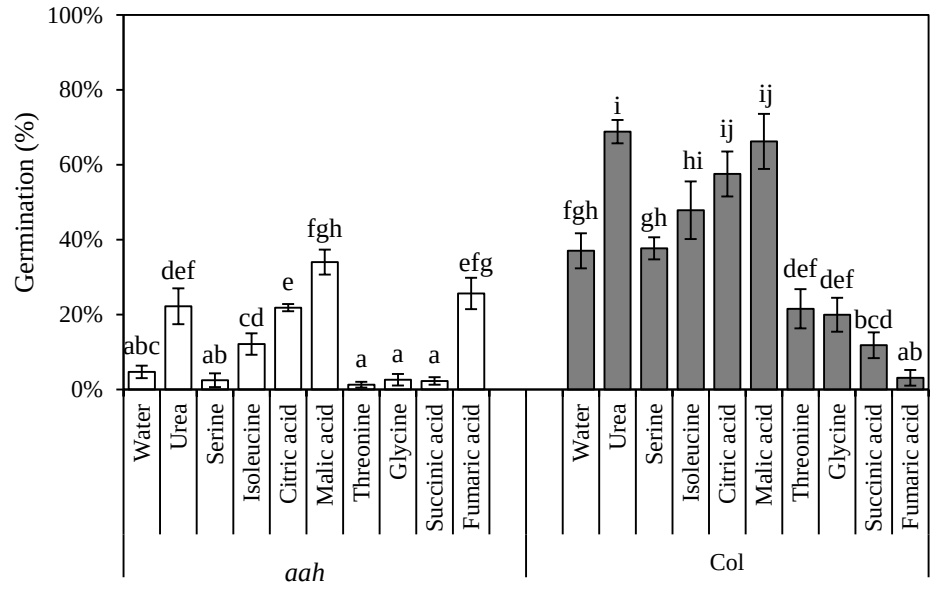


Figure 3.4 Amino acids (10 mM for each) feeding germination of *aah* mutant and Col matured in 5 mM nitrate solution. Means of maximum germination percentage from four replicates are presented. Error bars represent standard errors, and different letters indicate statistical significant differences (Student t-tests; $P < 0.05$)

ABA levels were measured in dormancy seeds (dry) that were developed in three concentrations of nitrate solutions. Our result showed that mutant seeds matured in low nitrate condition had much lower ABA content compare to wild type seeds, while seeds matured in control and high nitrate contained similar amount of ABA (Figure 3.5).

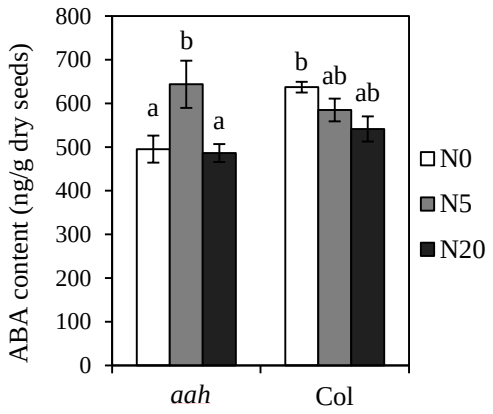


Figure 3.5 ABA content determination of *aah* mutant and Col seeds matured in different nitrate levels. Means of ABA content from four replicates are presented. Error bars represent standard errors, and different letters indicate statistical significant differences (Student t-tests; $P < 0.05$)

Expression analysis was performed on some genes close to *AAH* in purine pathway to investigate how seed response to ammonia lacking condition when *AAH* was deleted. *AAH* gene (Figure 3.6-A), which was knocked out from mutant seeds, had no expression on mutant seeds as was expected, but had similar expression levels in WT seeds matured in different concentrations of nitrate. Figure 3.6-B shows that mutant seeds matured in low nitrate solution had a higher relative *ALN* expression level among all treatments. For mutant seeds, the expression of *URE* (Figure 3.6-C) was found higher in low nitrate mature condition compare to other two nitrate levels, which was similar to the expression of that in WT seeds matured in all three levels of nitrate solution. The other two genes, *UGLYAH* and *UAH* (Figure 3.6 D, E), had similar relative expression levels for both types of seeds matured in all three nitrate levels.

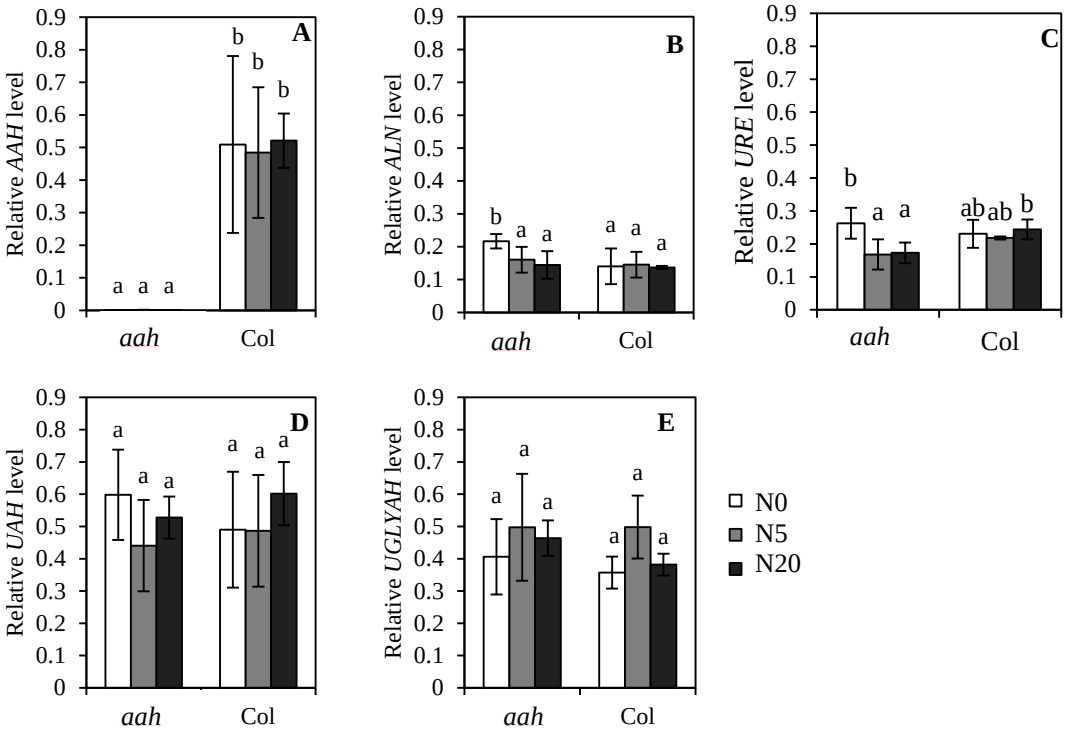


Figure 3.6 Relative expression level of genes in purine pathway relate to allantoate degradation: *AAH* (A), *ALN* (B), *URE* (C), *UAH* (D) and *UGLYAH* (E). Plant material was seeds of Col and *aah* mutant matured in different nitrate levels. Means of relative expression level (reference gene At3g25800) from four replicates are presented. Error bars represent standard errors, and different letters indicate statistical significant differences (Student t-tests; $P < 0.05$)

4. Discussion

Allantoate amidohydrolase is involved in the final steps of purine degradation, which releases two molecules of ammonia (and CO_2) directly without a urea intermediate (Todd and Polacco, 2006; Zrenner *et al.*, 2006). Ammonium was found to be required for nitrogen recycling in warm-climate legumes, also important for plant nitrogen supply under nitrogen-limiting conditions (Werner *et al.*, 2008). How plant compensates low nitrate condition and how seeds from different conditions of parent plants perform are interesting to be investigated. Our studying using an *aah* knock-out mutant and wild type (Col) Arabidopsis plants, applied with three concentrations of nitrate solution during their seed maturation stage, providing a brief insight in the effect of both internal ammonia production and external nitrogen compounds supply on seed dormancy.

Applying high amounts of nitrate during seed maturation did not decrease the seed dormancy of the *aah* mutant, and mutant seeds harvested from the low nitrate treatment showed enhanced dormancy. Moreover wild type seeds matured in different nitrate level had similar dormancy levels, which anyway show the involvement of nitrate in high dormancy of the mutant (Figure 3.1). It is known that nitrate assimilation of parent plants affect the accumulation of nitrate in seeds, and nitrate accumulation lower the requirement of GAs for germination (Alboresi *et al.*, 2005). Thus a higher dormancy of mutant seeds matured in no nitrate treated condition might be caused by the lower accumulation of N content in seeds, which could be determined by measuring N contents in seeds. Feeding experiment with KNO_3 showed that this compound can promote the germination in the mutant and not in the wild type. This finding suggests that missing ammonia produced by this enzyme can be one of the reasons for increased dormancy of the mutant. On the other hand, feeding with other nitrogen compounds (NH_4NO_3 and NH_4Cl) also increased the germination rate of *aah* seeds matured in low nitrate condition, but had less promoting or even a negative effect on seeds treated with 5 and 20 mM nitrate during maturation. This might due to the toxic effect of the excess nitrogen, most likely by ammonium (Figure 3.3). Another nitrogen compound, NH_4OH, was also applied in feeding experiment, however one day after seed incubation the seeds colour became darker than non-treated seeds and no seed germination was observed. Thus the lower germination percentage of seeds treated with ammonium compounds might cause by the toxicity of gaseous ammonia and/or ammonium salts, as ammonia is found inhibiting respiration of barely roots, and some other plant species (Vines and Wedding, 1960). Watanabe *et al.* (2014) suggested the role of allantoin in the modulating the stress response through ABA, where allantoin enhances ABA levels in plants. This agrees with our result that *aah* seeds matured in low nitrate condition showed a higher ABA level. We speculate that this is due to the fact that allantoinase is up regulated in the seeds that were grown in low nitrate condition (Hanzi He, unpublished) .Allantoinase acts on allantoin metabolite which enhances abiotic stress tolerance through synergistic activation of ABA metabolism (Watanabe *et al.*, 2014).

The feeding experiment was also performed with amino acids on dormant seeds. Our result showed that urea, malic acid and fumaric acid could release dormancy of *aah* seeds. Additionally, fumaric acid was observed inhibiting seed germination in wild type while it was promoting seed germination in mutant. Tschoep *et al.* (2009) found low accumulation of fumarate or malate in Arabidopsis leaves when the plants were grown on low nitrate and/or ammonium, and these two compounds maintained pH during the day in leaves when nitrate reduced to ammonium. Thus the lower germination percentage of Col seeds when fumaric acid was applied might be caused by the more acidic environment this compound provided. They also suggested that fumarate accumulation in *Arabidopsis* may induce plant growth in nitrogen abundant conditions, hence seeds matured in nitrate lacking condition might show different germination patterns in amino acids, which will be interesting to be investigated in the future. In the metabolite analysis malic acid and fumaric acid showed relatively lower abundance in mutant compare to wild type seeds, which might be another reason for higher dormancy of the mutant that could be compensated by addition of these compounds.

In the purine pathway, the higher expression of *ALN* (Figure 3.6-B) and *URE* (Figure 3.6-E) in seeds matured in low nitrate indicated the feedback of *aah* mutant to the reduction of ammonia. The activation of *URE* results in nitrogen assimilation from high accumulated metabolite (urea) in the mutant seeds. The activation of this gene might act as an ‘emergency response’ or an alternative pathway to produce ammonia when in nitrogen lacking condition.

In conclusion, this research showed that high dormancy of *aah* mutant can be compensated by feeding with nitrate compound (KNO_3), regardless of nitrate condition during seed maturation. Nevertheless mutant seeds matured in low nitrate showed higher dormancy which somehow shows the importance of nitrate during seed maturation for induction of dormancy in the mutant. Providing enough nitrate during seed maturation did not release seed dormancy, but effectively reduce the sensitivity of the mutant seed in saline stress, probably through ABA metabolism and signalling. The fact that applying fumaric acid during seed germination result in the same germination with none treated wild type suggest that low abundant of this metabolite probably has a role in higher dormancy of the mutant compare to wild type, especially regarding to the fact that wild type showed the opposite response to the application of fumaric acid in germination, which was obviously reduced compare to other amino acids or urea. Based on gene expression analysis, some of the genes showed higher expression to deal with low availability of ammonia caused by defect in the gene *aah*. In further research, the nitrogen content in seeds matured in different nitrate conditions is suggested to be determined, to have a better view of nitrogen accumulation from parent plants to seeds. Moreover, the seeds produced by plants grown in low nitrate conditions could be applied with amino acids to test the effect of urea, malic acid and fumaric acid on seed dormancy. Some improvements on gene analysis research, such as including more reference genes, should be applied in the future to ensure the reliability of the result.

5. Appendices

Appendix I

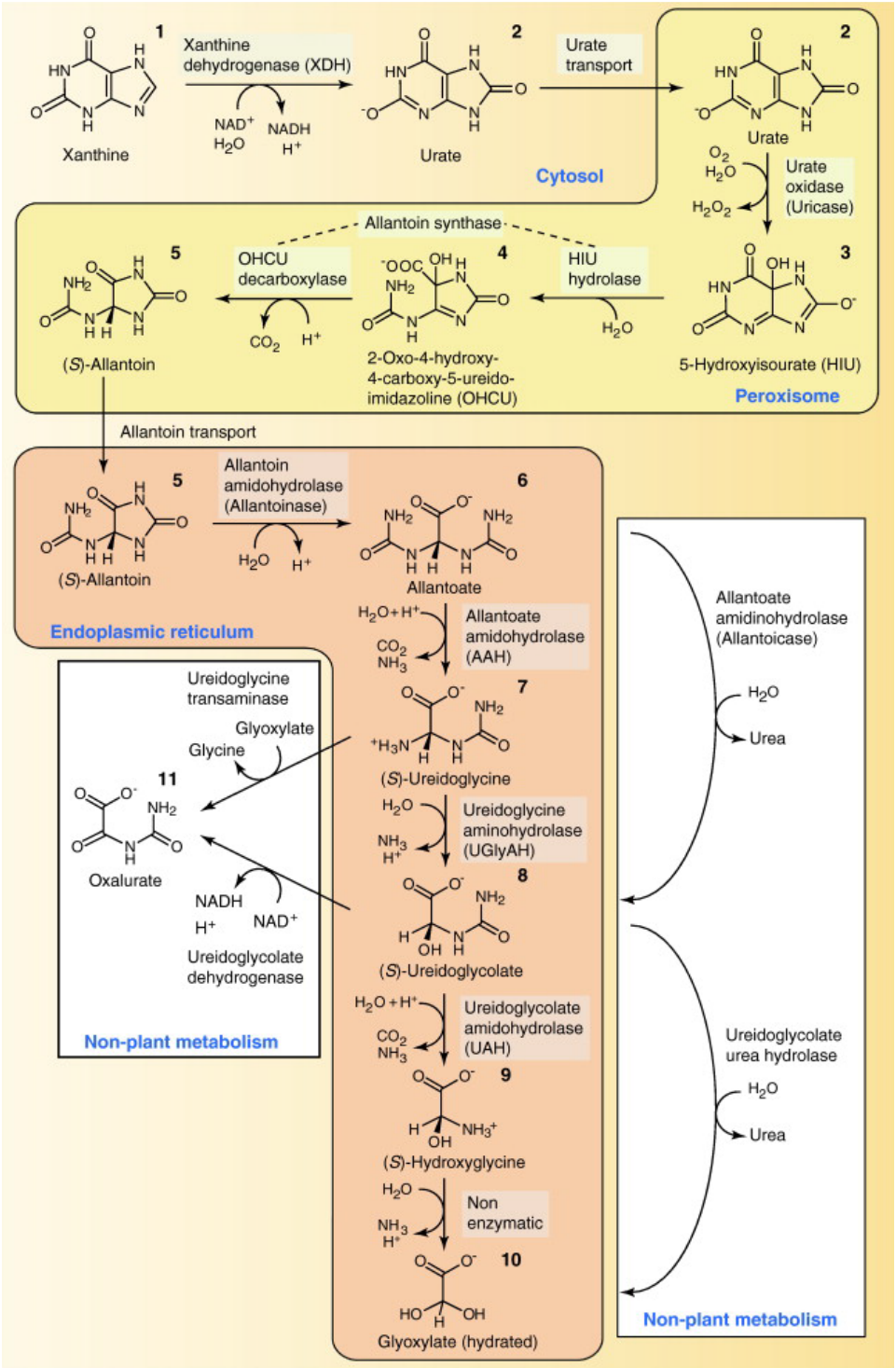


Figure 5.1 The catabolism of the purine ring system. The catabolic route is drawn beginning with xanthine, the first common intermediate in the degradation of all purine nucleotides. This figure is obtained from a paper of Werner and Witte (2011).

Appendix II Standard nutrient solution for plants

Table 5.1 Element concentrations in the standard nutrient solution

	Macro-element (mM)								Micro-element (μM)					
Element	N	K	Ca	Na	Mg	Cl	S	P	Fe	Mn	Zn	B	Cu	Mo3.5
Concentration	5	2.9	2	0.4	0.5	0.4	1.1	0.5	3.5	2.6	3.0	20	6.6	0.2

The pH of the solution was 7.0; EC=2.0; the solution is prepared by Unifarm (Bornsesteeg 4, 6708 PE Wageningen).

6. References

Alboresi A, Gestin C, Leydecker MT, Bedu M, Meyer C, Truong HN. 2005. Nitrate, a signal relieving seed dormancy in *Arabidopsis*. *Plant Cell Environ* **28:** 500-512.

Bewley JD. 1997. Seed germination and dormancy. *Plant Cell* **9:** 1055-1066.

Bewley JD, Hilhorst HWM, Bradford K, Nonogaki H 2013. *Seeds: physiology of development, germination and dormancy (3rd Edition)*: Springer, New York Heidelberg Dordrecht London.

French JB, Ealick SE. 2010. Biochemical and structural characterization of a ureidoglycine aminotransferase in the *Klebsiella pneumoniae* uric acid catabolic pathway. *Biochemistry* **49:** 5975-5977.

Huang X, Schmitt J, Dorn L, Griffith C, Effgen S, Takao S, Koornneef M, Donohue K. 2010. The earliest stages of adaptation in an experimental plant population: strong selection on QTLS for seed dormancy. *Mol Ecol* **19:** 1335-1351.

Hurtado PX, Schnabel SK, Zaban A, Vetelainen M, Virtanen E, Eilers PHC, van Eeuwijk FA, Visser RGF, Maliepaard C. 2012. Dynamics of senescence-related QTLs in potato. *Euphytica* **183:** 289-302.

Joosen RV, Kodde J, Willems LA, Ligterink W, van der Plas LH, Hilhorst HWM. 2010. GERMINATOR: a software package for high-throughput scoring and curve fitting of Arabidopsis seed germination. *Plant J* **62:** 148-159.

Maia J, Dekkers BJ, Provart NJ, Ligterink W, Hilhorst HW. 2011. The re-establishment of desiccation tolerance in germinated *Arabidopsis thaliana* seeds and its associated transcriptome. *PLoS One* **6:** e29123.

Masclaux-Daubresse C, Daniel-Vedele F, Dechorgnat J, Chardon F, Gaufichon L, Suzuki A. 2010. Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. *Ann Bot* **105:** 1141-1157.

Todd CD, Polacco JC. 2006. *AtAAH* encodes a protein with allantoate amidohydrolase activity from *Arabidopsis thaliana*. *Planta* **223:** 1108-1113.

Tschoep H, Gibon Y, Carillo P, Armengaud P, Szecowka M, Nunes-Nesi A, Fernie AR, Koehl K, Stitt M. 2009. Adjustment of growth and central metabolism to a mild but sustained nitrogen-limitation in *Arabidopsis*. *Plant Cell Environ* **32:** 300-318.

Vines HM, Wedding R. 1960. Some effects of ammonia on plant metabolism and a possible mechanism for ammonia toxicity. *Plant Physiol* **35:** 820.

Watanabe S, Matsumoto M, Hakomori Y, Takagi H, Shimada H, Sakamoto A. 2014. The purine metabolite allantoin enhances abiotic stress tolerance through synergistic activation of abscisic acid metabolism. *Plant Cell Environ* **37:** 1022-1036.

Werner AK, Sparkes IA, Romeis T, Witte CP. 2008. Identification, biochemical characterization, and subcellular localization of allantoate amidohydrolases from *Arabidopsis* and soybean. *Plant Physiol* **146:** 418-430.

Werner AK, Witte CP. 2011. The biochemistry of nitrogen mobilization: purine ring catabolism. *Trends Plant Sci* **16:** 381-387.

Zhou R, Squires TM, Ambrose SJ, Abrams SR, Ross AR, Cutler AJ. 2003. Rapid extraction of abscisic acid and its metabolites for liquid chromatography-tandem mass spectrometry. *J Chromatogr A* **1010:** 75-85.

Zrenner R, Riegler H, Marquard CR, Lange PR, Geserick C, Bartosz CE, Chen CT, Slocum RD. 2009. A functional analysis of the pyrimidine catabolic pathway in *Arabidopsis*. *New Phytol* **183:** 117-132.

Zrenner R, Stitt M, Sonnewald U, Boldt R. 2006. Pyrimidine and purine biosynthesis and degradation in plants. *Annu Rev Plant Biol* **57:** 805-836.