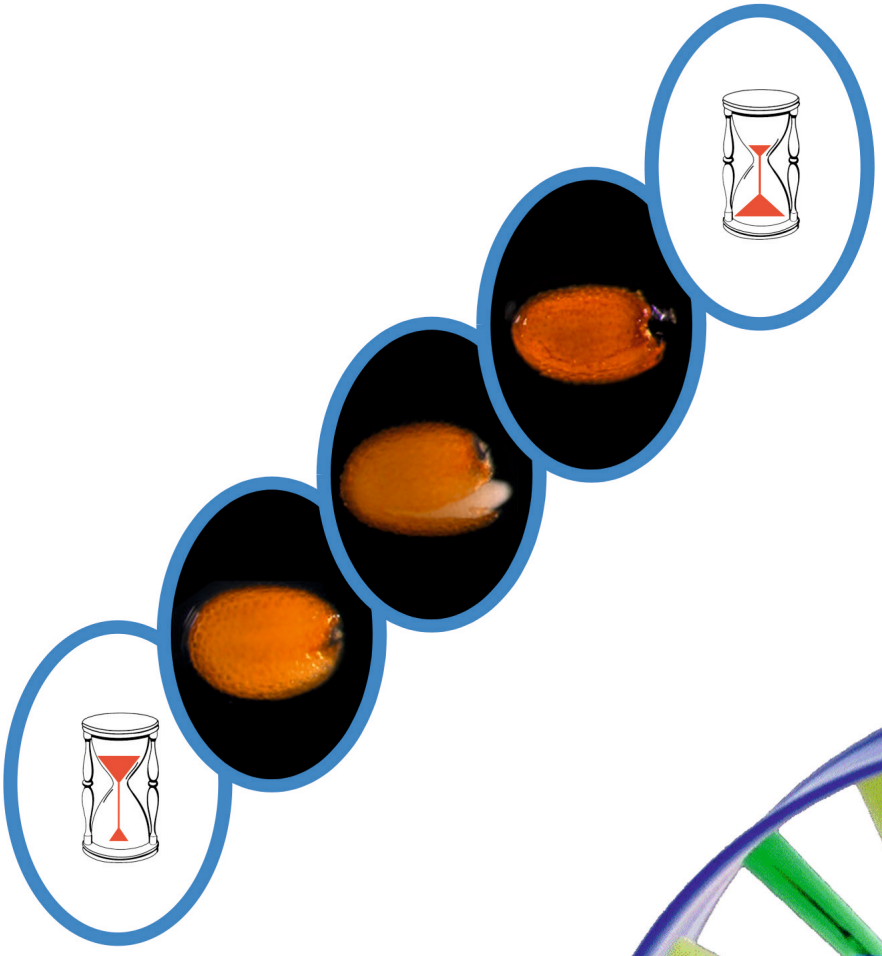
Identification and functional analyses of genes regulating seed dormancy, longevity and germination



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Identification and functional analyses of genes regulating seed dormancy, longevity and germination

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CHAPTER 1—General introduction

Farzaneh Yazdanpanah

Importance of seed

Seeds are a vital component of the world's diet since they contain high protein, starch and oil reserves. More than two thirds of the world population use seeds as a main source of food and nutrition. Seeds also provide the substrate for oil, medicine, fibre (e.g. cotton) and feeding for wide ranges of animals (Nambara and Nonogaki, 2012). Next to that, seeds are an important commodity and for this seeds with high performance are essential.

Seed characteristics

Beside their agricultural and economic significance, seeds are of immense ecological importance. They are dispersal units of higher plants that store the complete genetic information required for the regeneration of the species. Thus, successful seed germination is vital for a species to propagate itself. Traits such as seed dormancy, longevity and desiccation tolerance (DT) are important in this respect. Seed dormancy combined with DT ensures that germination will only occur at the appropriate time to optimize seedling establishment (Linkies et al., 2010), while DT and longevity are two essential traits for seeds to survive in the dry state (Waterworth et al., 2015). All these traits are established during seed maturation, the final stage of seed development. Seed development in higher plants can be roughly separated into three stages initiated by a double fertilization in the embryo sac. Following that, early morphogenesis of the diploid embryo and triploid endosperm occur through a series of cell divisions. In the second stage (early maturation), in parallel with cellular expansion, storage reserves, including proteins, starch and/or oil may accumulate in the embryo. Orthodox-seeded species acquire DT at this stage (seed filling). Subsequently, during the final stage (late maturation) seeds progressively acquire dormancy and longevity, metabolic activity declines to leave the seed entirely tolerant to desiccation while the final water content is reduced to around 7% (Olsen, 2001; Baud et al., 2002; Verdier et al., 2013). In the mature dry seeds of Arabidopsis, the embryo is surrounded by a single-cell endosperm layer and an outer layer of dead tissue of maternal origin (testa) (Belin and Lopez-Molina, 2010). Accumulation of flavonoid pigments in the testa give the mature seeds a brown colour.

i. Seed dormancy

Seeds are usually equipped with dormancy mechanisms to prevent preharvest sprouting and also to prevent germination during (temporarily) favourable conditions. (Bewley, 1997). Furthermore, dormancy allows seeds to spread germination over time, reducing the risk of losing an entire generation by catastrophe (e.g. late frosts, drought, herbivory) and plant species most probably survive (Hilhorst, 2007). In mature seeds, the release of dormancy may either occur gradually by seed dry storage (after-ripening) or low temperature treatment of imbibed seeds (stratification) (Bewley, 1997; Bewley et al., 2013). During after-ripening and stratification seeds regain their germination ability. After-ripening, just like cold stratification, widens the environmental conditions that permit germination. Seed dormancy can be quantified by determining the germination percentage during after-ripening or, more accurately expressed, by the days of seed dry storage (after-ripening) until 50% germination is reached (DSDS50; Alonso-Blanco et al., 2003).

The level of dormancy in seeds is determined by a number of factors, such as genetics, hormonal regulation and environmental factors functioning during development and maturation (Gutterman et al., 1975; Fenner and Thompson, 2005; Bewley, 2013).

Genetics and hormonal regulation of seed dormancy and germination. Although the environment is a key determining factor in depth of seed dormancy, endogenous factors, including genetic and hormonal control, are the main regulators. Abscisic acid (ABA) and gibberellins (GAs) are the major hormones regulating seed dormancy and germination, although roles for other hormones such as ethylene, auxin, brassinosteroid (BR), and strigolactone have also been suggested (Liu et al., 2013). ABA positively regulates dormancy and acts as a negative regulator of germination whilst GAs have the opposite role with the ABA/GA balance determining the developmental state of the seed (Finch-Savage and Leubner-Metzger, 2006). In many cases, changes in environmental conditions which influence seed dormancy or germination lead to alterations in the expression of genes involved in ABA- or GA-metabolism (Seo and Koshiba, 2002).

In Arabidopsis and some other species, mutant approaches have been proven extremely useful tools to reveal the roles of ABA, GAs and other important

regulators of seed dormancy which are involved in hormone-independent pathways (Koornneef and Karssen, 1994). For instance, mutants in genes regulating ABA levels (e.g 9-*cis*-epoxycarotenoid dioxygenase (*NCED*) family and cytochrome P450 CYP707A family) or -sensitivity (e.g. *ABSCISIC ACID INSENSITIVE 3-5*) display a reduced degree of seed dormancy (Koornneef et al., 1982; Koornneef et al., 1984). Moreover, severe mutants defective in GA-biosynthesis genes (*GA1*, *GA2* and *GA3*) fail to germinate without exogenous GA, indicating that GAs are absolutely required for germination (Koornneef and Van der Veen, 1980).

Along with *ABI3-1*, other major regulators of seed maturation *LEAFY COTYLEDON 1* and *2* (*LEC1* and *LEC2*; Meinke et al., 1994) and *FUSCA 3* (*FUS3*; Bäumlein et al., 1994) have been identified by mutants that display non-dormant phenotypes with defective seed maturation (Raz et al., 2001), indicating that dormancy is part of the maturation phase of seed development.

In addition to those genes that are involved in hormonal action, there are also many other important regulators of seed dormancy which are involved in hormone-independent pathways. Two examples are *HISTONE MONOUBIQUITINATION1* (*HUB1*) and *REDUCED DORMANCY2* (*RDO2*). These genes were identified in the same mutagenesis screen on the basis of reduced dormancy. *HUB1* was shown to encode a C3HC4 Ring finger protein involved in the mono-ubiquitination of histone H2B that regulates initiation and early elongation steps in transcription. *RDO2* is allelic to the recently identified dormancy gene TFIIS, which is a transcription elongation factor. These finding suggests that *RDO2* and *HUB1* are involved in the same process and reveal a role for chromatin modelling in seed dormancy (Liu et al., 2007).

Another screen for mutants with reduced seed dormancy led to the identification of the *delay of germination 1* (*dog1*) mutant (Bentsink et al., 2006). *DOG1* had been already identified as a major QTL (Quantitative Trait Locus) for seed dormancy variability among natural Arabidopsis accessions (Alonso-Blanco et al., 2003; Bentsink et al., 2010). *DOG1* is a member of a small gene family of un-known molecular function that is highly expressed during seed maturation. Both transcript level and protein abundance of *DOG1* in freshly harvested seeds showed a strong correlation with dormancy levels (Bentsink et al., 2006; Nakabayashi et al., 2012).

***Regulation of seed dormancy by environmental factors*.** A range of environmental signals, such as temperature, light, water and nutrients affect seed dormancy. However, the responses of seeds to these factors are different. For example, low temperatures during seed maturation increase primary dormancy in Arabidopsis, while warm temperatures have the opposite effect (Schmuths et al., 2006; Chiang et al., 2009; Donohue, 2009; Kendall et al., 2011). In contrast, during seed imbibition, as described above, low temperature (cold stratification) relieves dormancy. Light is another environmental cue regulating seed dormancy and germination. The effect of photoperiod during seed maturation on germinability varies in different species. In Arabidopsis, long days induce low dormancy levels, whereas short days induce high dormancy levels (Munir et al., 2001). Light also stimulates the seed germination of most annual plant species (Kyereh et al., 1999). Nitrate is an important nitrogen source for plants and can act as a signaling molecule that controls seed dormancy. In Arabidopsis exogenous nitrate reduces dormancy when provided to the mother plant during seed development and promotes germination when applied to dormant seeds (Alboresi et al., 2005).

ii. Seed longevity

Seed longevity is defined as the germination ability of seeds after long-term storage. Seed germination ability consists of an after-ripening and an aging phase. During after-ripening, seeds release dormancy and gain full germination ability but, subsequently, due to deterioration processes, germination rate is gradually reduced to a complete loss of germinability at later periods of storage (Bernal-Lugo and Leopold, 1995; Bewley et al., 2012). Several factors influence the rate of the deterioration process during seed maturation and storage. During seed maturation, the maternal environment such as temperature, light and humidity may affect the extension of longevity (Ellis and Roberts, 1980; Nagel and Börner, 2010). Factors that are involved in seed dormancy release during dry storage might also promote seed aging, as is the case for oxidation, (Baskin and Baskin, 1998). Although (limited) oxidation seems beneficial for dormancy release, increased oxidative stress due to the accumulation of reactive oxygen species (ROS) during storage, induces seed deterioration and, consequently, reduces seed longevity. The accumulation of ROS leads to oxidation of lipids, proteins, and genetic material (DNA and RNA) (Møller et al., 2007). To counteract oxidative damage, dry mature seeds are equipped with protecting

mechanisms (antioxidant systems) to avoid, scavenge, and neutralize reactive molecules (Blokhina et al., 2003).

Seed longevity can be assessed by natural and artificial aging. Due to slow natural aging, artificial aging methods were established to predict seed longevity in a short period of time. In most commonly used artificial aging protocols, seeds are stored at high temperature and high relative humidity for a short period, after which seed viability is evaluated by a germination assay (Lehle and Tanner, 1973; Hampton and TeKRONY, 1995). This method is presumed to mimic natural dry aging (Tesnier et al., 2002; Rajjou et al., 2008).

iii. Seed performance

The physiological potential of seeds to germinate under various environmental situations is referred to as seed performance. The performance of seeds is determined by a combination of factors such as physical properties, genetic homogeneity, uniformity, vigour, viability, dormancy and longevity (Basra, 2006). Assessment of seed performance can be performed by measuring dormancy level (DSDS50) and germination under different conditions, such as osmotic stress (mannitol), salt stress (NaCl), abscisic acid (ABA), temperature stress and artificial aging conditions. For such analyses maximum germination percentage (Gmax) at the end of the germination assay is usually used. However, by using the Germinator tool developed by Joosen et al. (2010), it is possible to extract other parameters from the germination assay, including the rate of germination that enable to do the analysis more accurately. The Germinator package is a software for efficient automatic scoring and evaluation of germination. It enables to calculate the rate of germination with three different variables: the time required for 10% and 50% germination of viable seeds, referred to as t10 and t50 respectively and the uniformity of germination, U8416, defined as the time interval between 84% and 16% of viable seeds to germinate (Joosen et al., 2010). Rate of germination is usually used for data analysis, especially for seed dormancy and longevity that are two main determinants of seed performance. Regarding seed longevity, reduction in the rate of germination is the first indicator of deterioration and a marker for the initiation of the aging process (Bernal- Lugo & Leopold, 1992; Bewley et al., 2013). The rate of germination can also be used to better distinguish small dormancy differences between genotypes. Furthermore, low dormancy does not

necessarily lead to fast and uniform germination and those two characteristics should be determined by germination rate parameters.

In agricultural crops seed dormancy generally is not a desirable characteristic; instead rapid and uniform germination and growth are of central importance. Several methods have been developed to obtain these characteristics and enhance the seed performance. Seed priming is the most widely used one; a pre-sowing treatment which partially hydrates the seed, then dehydrates them and is followed by dry storage until sowing (McDonald, 2000; Bewley, 2013). Although a priming treatment mainly improves seed performance it has a negative effect on their storability. Therefore, understanding the molecular mechanisms that control seed dormancy, longevity and germination may help to competently improve seed performance and efficient seed germination.

Arabidopsis as a reference plant

Applied research is usually carried out on crop plants, while basic molecular and genetic studies often concentrate on the model plant *Arabidopsis thaliana*. Arabidopsis has many advantages for genetic and genomic research including a short life cycle compared to most crop plants, high seed production by self-pollination, space-saving cultivation, wide geographic and environmental distribution, relatively small genome size with known sequence (Initiative, 2000), the availability of numerous mutants in the stock centers (https://www.arabidopsis.org/), as well as efficient and well established methods of genetic transformation (Meyerowitz, 1989; Meyerowitz et al., 1991; Pyke, 1994). Moreover, information gained on Arabidopsis likely can be transferred to most higher plant species, especially the major crops of the world, to be efficiently used for plant improvement programs. However, whether this is the case has to be investigated for every specific situation.

Scope of the thesis

Knowledge of the genes affecting seed performance is very useful since it can be used in breeding programs to improve seed quality of cultivars. The traits that contribute to seed performance are complex adaptive traits influenced by interactions among multiple genetic- and environmental factors (Koornneef et al., 2002). Therefore, QTL analysis is a common approach to identify genes involved in those phenotypes. Several QTLs for seed performance have been identified,

especially for seed dormancy (Alonso-Blanco et al., 2003; Bentsink et al., 2010) and longevity (Bentsink et al., 2000; Clerkx et al., 2004; Nguyen et al., 2012), which are two important traits affecting seed performance. Furthermore, Joosen et al. (2012) found several QTLs for many seed germination characteristics. However, information regarding the underlying genes of these QTLs is scarce. In QTL analysis, near isogenic lines (NILs) are usually used to confirm the presence and the effect of a QTL. Transcriptomic analysis of such NILs can potentially result in distinctive gene expression profiles that allow the identification of critical genes involved in seed performance and provide candidate target genes for further characterization.

The objective of this study is to reveal genetic regulators for seed dormancy and possibly other traits related to seed performance. For this purpose, we have first investigated the transcriptomes of freshly harvested (dormant) and after-ripened (AR; non-dormant) 24-hour imbibed seeds of four different *DELAY OF GERMINATION* near isogenic lines (*DOG*NILs) and the Landsberg *erecta* (L*er*) wild type with varying levels of primary dormancy. This comparative microarray led to the identification of the genes differentially expressed in all genotypes tested. In a next step, T-DNA knock-out lines of identified differentially expressed genes were investigated for their seed performance phenotype (**Chapter 2**). Knock-out mutants in several genes showed dormancy and germination-related phenotypes. Among these, three mutants displaying the most interesting phenotypes were selected for in depth studies of their corresponding genes. In **Chapter 3,** *FAST GERMINATING SEEDS* (*FGS*), a novel gene whose lack of function mutant displays reduced seed dormancy, faster and more uniform germination and lower sensitivity to abiotic stress was further investigated using metabolomics, proteomics and genetic analysis. In **Chapter 4,** I study the role of the *NADP-MALIC ENZYME 1* (*NADP-ME1*) gene in seed longevity, since loss-of-function of NADP-ME1 resulted in reduced seed viability relative to wild type. In this chapter, by combining biochemical and physiological methods, I investigate the participation of NADP-ME1 in seed germination and seed aging. The last gene I study is *ALLANTOATE AMIDOHYDROLASE* (*AtAAH*) that encodes an enzyme in the uric acid catabolic pathway. Seeds mutated in *AtAAH* display increased seed dormancy. Since AtAAH is a key enzyme in the pathway that produces usable nitrogen for subsequent anabolic reactions, I hypothesized that higher dormancy of the *Ataah* mutant seeds is the consequence of a defective nitrogen

production. To test this hypothesis, exogenous nitrate was applied during seed maturation and seed germination to see whether it can rescue the germination behaviour of the mutant (**Chapter 5**). In **Chapter 6**, the findings presented in this thesis are discussed.

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Chapter 2 — Differentially expressed genes during the imbibition of dormant and after-ripened seeds– a reverse genetics approach

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ABSTRACT

Background: Seed dormancy, defined as the incapability of a viable seed to germinate under favourable conditions, is an important trait in nature and agriculture. Despite extensive research on dormancy and germination, many questions about the molecular mechanisms controlling these traits remain unanswered, likely due to its genetic complexity and the large environmental effects which are characteristic of these quantitative traits. To boost research towards revealing mechanisms in the control of seed dormancy and germination we depend on the identification of genes controlling those traits.

Methods: We used transcriptome analysis combined with a reverse genetics approach to identify genes that are prominent for dormancy maintenance and germination in imbibed seeds of *Arabidopsis thaliana*. Comparative transcriptomics analysis was employed on freshly harvested (dormant) and after-ripened (AR; non-dormant) 24-hour imbibed seeds of four different *DELAY OF GERMINATION* near isogenic lines (*DOG*NILs) and the Landsberg *erecta* (L*er*) wild type with varying levels of primary dormancy. T-DNA knock-out lines of the identified genes were phenotypically investigated for their effect on dormancy and AR.

Results: We identified conserved sets of 45 and 25 genes which displayed higher expression in seeds of all dormant and all after-ripened *DOG*NILs and L*er*, respectively. Knock-out mutants in these genes showed dormancy and germination related phenotypes.

Conclusions: Most of the identified genes had not yet been implicated in seed dormancy or germination. This research will be useful to further decipher the molecular mechanisms by which these important ecological and commercial traits are regulated.

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INTERODUCTION

Freshly matured seeds usually exhibit primary dormancy, a trait defined as the failure of viable seeds to germinate under favourable conditions (Bewley 1997). Seed dormancy plays a crucial role in the survival of plant species, but is also important for agricultural practice to prevent pre-harvest sprouting under cool, high humidity conditions (Gubler *et al.* 2005). Primary dormancy can be released by either cold stratification, which is a low-temperature treatment of imbibed seeds, or by an extended period of dry seed storage (after-ripening; AR) (Bewley *et al.* 2012).

The transition from dormancy to germination is a critical step in the life cycle of plants (Holdsworth *et al.* 2008a). The plant hormone abscisic acid (ABA) has long been known to play a major role in the establishment and maintenance of seed dormancy and the inhibition of seed germination, whereas gibberellins (GAs) and several other hormones, including brassinosteroids, ethylene, and cytokinins, have been shown to promote seed germination (Liu *et al.* 2013). However, it is especially the balance between ABA and GA that controls the decision to either germinate or not (Finch-Savage and Leubner-Metzger 2006). Mutations in genes regulating ABA levels or -sensitivity result in a reduced degree of seed dormancy (Koornneef *et al.* 1982, Koornneef *et al.* 1984). Whereas GA biosynthesis or sensing mutants result in a block of germination (Koornneef and van der Veen,1980, Griffiths *et al.* 2006, Iuchi *et al.* 2007, Yamauchi, 2008). This hormonal control is also integrated with the seed's responses to environmental conditions, such as light (Oh *et al.* 2009), temperature (Toh *et al.* 2008, Yamauchi *et al.* 2004) and nutrients (Matakiadis *et al.* 2009).

Recent advances in gene expression analysis using microarrays allow genome-wide expression studies to characterize seed dormancy and germination (Cadman *et al.* 2006, Fu *et al.* 2005, Gallardo *et al.* 2001, Holdsworth *et al.* 2008b, Howell *et al.* 2009, Le *et al.* 2010, Nakabayashi *et al.* 2005, Sreenivasulu *et al.* 2008). Carrera *et al.* (2008) used a targeted transcriptomics approach in imbibed non-dormant mutants (*aba1* and *abi1*) compared to wild-type seeds that were or were not after-ripened. They concluded that, in Arabidopsis, after-ripening and dormancy are controlled by genetically separate pathways, and that ABA only affects the induction and maintenance of dormancy in imbibed seeds, but not after-ripening. The work

also showed that application of exogenous ABA to after-ripened seeds does not mimic dormant seed states with respect to gene expression profiles. Recently it was shown that seed dormancy maintenance in the imbibed state was mainly controlled at the transcriptional level (Bai *et al*, 2017) and that transcriptional differences between dormant and non-dormant seed become visible already at early imbibition (Dekkers *et al.* 2016a, Preston *et al.* 2009).

Despite extensive research on dormancy and germination, many questions about the molecular mechanisms controlling these traits remain unanswered, likely due to its genetic complexity and the large environmental effects which are characteristic of these quantitative traits. Employing whole-genome scans for quantitative trait loci (QTL) is a common approach to identify genes involved in complex phenotypes. Particular attention in this method is given to the role of natural variation in the regulation of traits related to plant adaptation. Natural variation has been used to identify loci that control seed dormancy in nature. QTL analyses on six Recombinant Inbred Line (RIL) populations have identified eleven *DELAY OF GERMINATION* (*DOG*) QTL of which nine have been confirmed by near isogenic lines (NILs). The different *DOG* loci affect dormancy mainly by distinct genetic pathways as was concluded from the absence of strong epistatic interactions in the QTL analysis. This finding was confirmed by transcriptome analyses in freshly harvested dry seeds of the main *DOG*NILs, these lines showed distinct expression patterns compared to their genetic background Landsberg *erecta* (L*er*). The genes identified in the different *DOG*NILs represent largely different gene ontology profiles (Bentsink *et al.* 2010).

Here we aim at identifying genes that are required for dormancy maintenance and germination of imbibed seeds. Moreover, we focus on what is in common between the different pathways. For this the transcriptome of freshly harvested (dormant) and after-ripened (AR; non-dormant) 24-hour imbibed seeds of the same set of *DOG*NILs and L*er* was investigated. We have identified sets of 45 and 25 genes that were up-regulated in seeds of all dormant (D-up) and all after-ripened (AR-up) *DOG*NILs and L*er*, respectively. We have investigated their role in seed performance by analysing knock-out (KO) mutants in these genes. With seed performance we refer to the capacity of seeds to germinate under various

environmental conditions. Traits that contribute to seed performance are seed dormancy, seed longevity (as estimated in an accelerated aging test) and germination under stress conditions, such as high salt, osmotic stress and ABA treatment (Joosen *et al*, 2013). In this study we have characterised several genes affecting seed performance.

MATERIAL AND METHODS

Plant Material

The near isogenic lines of four *DELAY OF GERMINATION* (*DOG*) loci; *NILDOG1*-Cvi, *NILDOG2*-Cvi, *NILDOG3*-Cvi and *NILDOG6*-Kas-2 and Landsberg *erecta* (L*er*) were earlier described by Bentsink *et al*. (2010). Although for some of the *DOG* loci several NILs, containing introgression fragments from different accessions, were available we have chosen the ones with the strongest phenotypic effects. T-DNA insertional mutant lines and Columbia-0 (Col-0; N60000) were ordered from the European Arabidopsis Stock Center (NASC, http://arabidopsis.info/). Details (SALK/SAIL entry, AGI code, knock out number and encoded protein) of T-DNA lines are provided in Table S1.

Growth conditions

NILs: Seeds were sown in Petri dishes on water-saturated filter paper, followed by a 4-day cold treatment at 4°C, and transferred to an acclimated room at 25°C with 16h light/8h dark for 2 days before planting in 7-cm pots with standard soil. Plants were grown in an air-conditioned greenhouse at 70% relative humidity, supplemented with additional light (model SON-T plus 400W, Philips, Eindhoven, The Netherlands) providing a day length of 16h light (long day), with light intensity 125 mmol m^{-2} s^{-1}, and maintained at a temperature of 22–25°C (day) and 18°C (night). NILs were grown in a randomized complete block design with eight replicates. An experimental plot consisted of a row of 12 plants. At harvest the seeds of eight plants were bulked. Three of the eight replicates were used for the microarray analyses.

T-DNA knock-out lines: Lines were screened for homozygous insertions and grown with the wild type Columbia (Col) under greenhouse conditions using Rock

wool supplemented with a Hyponex solution, in a randomized complete block design with four replicates per genotype.

Sample preparation for microarray analyses

Dormant seeds were imbibed for 24h in continuous light at 22°C and then stored at -80°C until RNA isolation. After-ripened seeds were imbibed for 24h under the same conditions as the dormant seeds as soon as the seeds reached 100 percent germination in the germination experiment, also these seeds were stored at -80°C until RNA isolation.

Microarray analysis

Total RNA was prepared from 24-h imbibed seeds using RNAqueous columns with Plant RNA isolation aid (Ambion, Austin, TX, USA) according to the manufacturer's protocol. The RNA was further purified through precipitations with isopropanol and a high salt solution containing 0.24 M sodium citrate and 0.16 M sodium chloride and subsequently with 2 M lithium chloride. RNA was qualitatively assessed and quantified using an Agilent 2100 Bioanalyzer with the RNA 6000 Nano Labchip® kit (Agilent, Santa Clara CA, USA) and Nanodrop1000™ spectrometry (NanoDrop Technologies, Inc., Wilmington, DE, USA). RNA was processed and cRNA synthesized according to the 3' GeneChips OneCycle kit and hybridized on the ATH1 GeneChip (Affymetrix Inc., Santa Clara, CA, USA). The GeneChip data were analyzed using the R statistical programming environment and the Bioconductor packages (Irizarry *et al.* 2003, Smyth 2004, Team 2012). The data was normalized using the RMA algorithm and a linear model was fitted to the data for comparisons of dormant to after-ripened seed within each genotype, the empirical Bayes method was used to reduce the gene wise sample variance (Ritchie *et al*, 2015). The P values were then adjusted for multiple testing with the Benjamini and Hochberg method to control for false positives (Benjamini and Hochberg 1995). The microarray data were deposited in NCBI's Gene Expression Omnibus (GEO number GSE90162). Microarray quality and reproducibility data is presented in Fig. S1. Dormancy up regulated genes (D-up) represent genes up-regulated (P >0.0001) in the following comparisons, L*er* dormant vs L*er* after-ripened, NIL*DOG1* dormant vs NIL*DOG1* after-ripened, NIL*DOG2* dormant vs NIL*DOG2* after-ripened, NIL*DOG3* dormant vs NIL*DOG3* after-ripened, NIL*DOG6* dormant vs NIL*DOG6* after-ripened

and vice versa After-ripening up regulated genes (AR-up) represent genes up-regulated (P >0.0001) in the following comparisons, L*er* after-ripened vs L*er* dormant, NIL*DOG1* after-ripened vs NIL*DOG1* dormant, NIL*DOG2* after-ripened vs NIL*DOG2* dormant, NIL*DOG3* after-ripened vs NIL*DOG3* dormant, NIL*DOG6* after-ripened vs NIL*DOG6* dormant.

T-DNA knock-out genotype analyses

A quick isolation method modified from (Cheung *et al.* 1993) was performed to extract genomic DNA from leaves. In short, samples were ground in an extraction buffer containing 2 M NaCl, 200 mM Tris-HCl (pH 8), 70 mM EDTA and 20 mM Na2S2O5. The grinding was conducted with a stainless steel ball at 30 Hz for 1 min (96-well plate shaker, Mo Bio Laboratory). Then samples were incubated at 65°C for 1 h. Supernatants were collected after centrifugation at maximum speed for 10 min. DNA was precipitated by adding iso-propanol and 10M NH4Ac with ratio of 1:1/2:1 to the supernatant. This mixture was incubated at room temperature for at least 15 min, then centrifuged for 20 min at maximum speed. The DNA pellet was retrieved and rinsed with 70% ethanol followed by centrifugation for 5 min at maximum speed to recover the pellet. After drying, the DNA pellet was dissolved in distilled water. Homozygous T-DNA insertion lines were screened with gene specific primers (left and right) and insert border primers (Table S1). T-DNA plants that amplified only the insertion product were consider to be homozygous mutants.

Polymerase chain reactions (PCR) were performed in a 12.5 µL-volume containing approximately 30 ng DNA, 25 µM of each dNTP, 25 ng of forward and reverse primers, 0.05U of DNA polymerase (Firepol, Solis BioDyne), 312.5 µM of $MgCl_2$. The reaction protocol was as follows; denaturation at 95^{o}C for 5 min followed by 30s at 95^{o}C, 30s annealing at 52 to 57^{o}C and a 45s to 2 min extension at 72^{o}C, this cycle was repeated for 35 times, and ended with a final amplification for 10 min at 72^{o}C. The polymorphism was detected by agarose gel electrophoresis at concentrations from 1.5 % and higher (w/v) depending on size of differences.

Germination assays

Germination tests to follow the release of seed dormancy were performed as described by Alonso-Blanco *et al*. (2009) with small adjustments. In short, at several time intervals during seed dry storage until all seed batches reached 100 percent germination aliquots of 50 to 100 seeds of each genotype were evenly sown on a filter paper soaked with 0.7 ml demineralized water in a 6-cm Petri dish. Petri dishes were placed in moisture chambers consisting of plastic trays containing a filter paper saturated with tap water and closed with transparent lids. Chambers were stored for 1 week in a climate chamber illuminated with 38-W Philips TL84 fluorescent tubes at 8 W m2 in continuous light at 22°C. After that, the total number and the number of germinating seeds was scored and the percentage of germinating seeds was calculated.

Germination under stress conditions was performed on fully after-ripened seeds. Stress conditions were: osmotic stress (-1 MPa mannitol; Sigma-Aldrich), salt stress (130 mM NaCl; Sigma-Aldrich), ABA stress (0.15 µM ABA; Duchefa Biochemie). ABA was dissolved in 10 mM MES buffer (Sigma-Aldrich) and the pH adjusted to 5.8. To measure seed longevity, an accelerated aging test was performed by incubating seeds above a saturated ZnSO4 solution (40°C, 85% relative humidity) in a closed tank for 5 days. Then the seeds were taken out and germinated on demineralized water as described before.

RESULTS

Identification of seed dormancy and after-ripening up-regulated genes

Seeds of L*er*, NIL*DOG1*-Cvi, NIL*DOG2*-Cvi, NIL*DOG3*-Cvi and NIL*DOG6*-Kas-2 were investigated for their dormancy status. After-ripening was followed by performing germination tests during a time course of dry seed storage (Figure 1a). After 120 days all genotypes had lost dormancy and showed 100% germination. NIL*DOG2* was less dormant and NIL*DOG3*, NIL*DOG6* and NIL*DOG1* were more dormant when compared to L*er*. Both dormant and after-ripened seeds of each genotype were sampled 24 hours after sowing (HAS) for microarray analysis, allowing the comparison of the dormant and after-ripened seed transcriptomes of these five genotypes with varying levels of primary dormancy.

The transcriptome data was investigated to identify genes that are up-regulated in 24h imbibed dormant (D) L*er*, NIL*DOG1*, NIL*DOG2*, NIL*DOG3* and NIL*DOG6* seeds and genes that are up-regulated in 24h imbibed after-ripened (AR; non-dormant) seeds of the same lines. 1896 genes ($P<0.0001$) were differentially expressed when performing within-genotype comparisons for the two stages analysed (dormant versus AR). In dormant seeds 63, 670, 198, 348, and 441 genes were up regulated and 49, 609, 148, 475 and 688 genes were up-regulated in AR seeds for L*er*, NIL*DOG1*, NIL*DOG2*, NIL*DOG3* and NIL*DOG6*, respectively (Figure 1b).

A large proportion of the differentially expressed genes is specific for the genotypes analysed at $P<0.0001$; however, these genes were differentially expressed in the other genotypes at lower significances. This has been visualised for the genes that are specific for NIL*DOG1* (Figure 1cd). Most of the 245 NIL*DOG1* D-up and 159 NIL*DOG1* AR-up genes are differentially expressed ($P < 0.01$) in the other genotypes. This indicates that the genes that are specifically differentially expressed are based on quantitative expression differences rather than qualitative.

Genes that are important for dormancy and AR are expected to be differentially expressed between these stages in all genotypes tested (intersections in the Venn diagrams of Figure 1b). This led to the identification of 45 up-regulated genes in all dormant genotypes (Dormancy-up; D-up; Table 1) and 25 genes that were up-regulated in all after-ripened genotypes (After-ripened–up; AR-up; Table 2). Further investigation of the expression patterns using the Seed EFP browser (http://www.bioinformatics.nl/efp/cgi-bin/efpWeb.cgi) revealed that, in general, all the genes that were up-regulated in dormant seeds at 24 HAI, were highly expressed in dry seeds, remained high during the imbibition of dormant seeds but were down regulated during the germination of AR seeds. Vice versa, genes that were up-regulated in AR seeds, had a low expression in dry seeds that increased with imbibition time (Figure S2). Furthermore, the relation with dormancy becomes clear when the expression of the individual genes is investigated in imbibed seeds of L*er*, *dog1-1* and NIL*DOG1*-Cvi that have very clear dormancy differences (Figure 1e). D-up genes are highly expressed in dormant L*er* and NIL*DOG1*-Cvi seeds, whereas AR-up genes are highly expressed in the non-dormant *dog1-1* mutant (Figure 1fg).

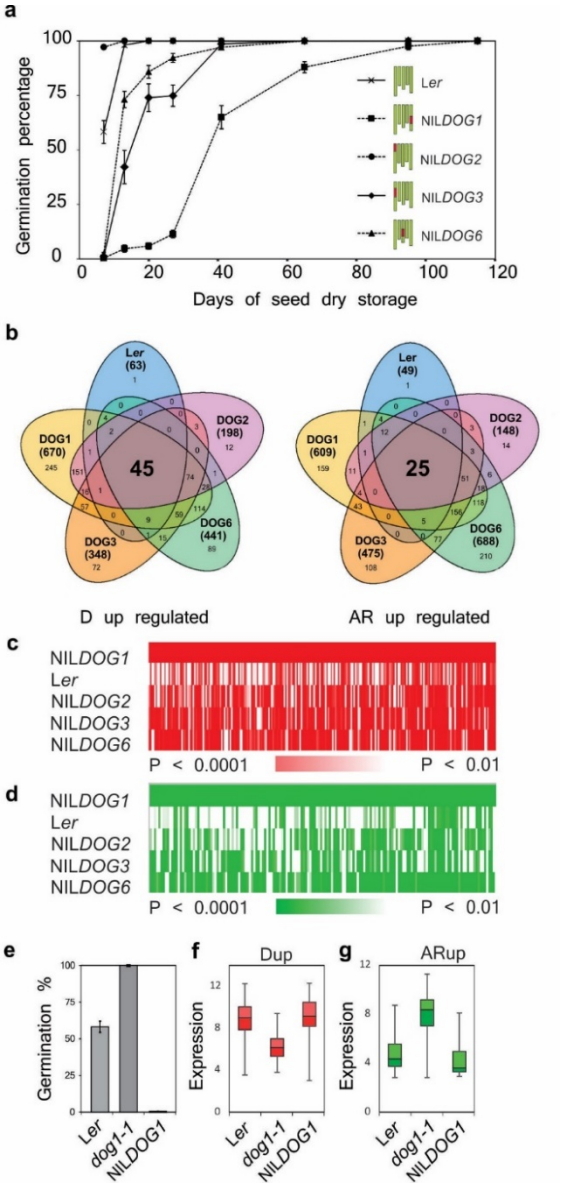

Figure 1. Microarray analysis of dormant and after-ripened seeds after 24h of imbibition of five genotypes with differing dormancy levels; L*er*, NIL*DOG1*, NIL*DOG2*, NIL*DOG3* and NIL*DOG6*. (a) After-ripening requirement of the five genotypes. On the right graphical representations of the NILs are depicted showing the 5 chromosomes with the introgressed regions (in red) in an otherwise L*er* background (in green). (b) Venn diagrams showing the number of genes that are differentially expressed ($P < 0.0001$) in dormant (D-up) and after ripened (AR-up) 24-hour imbibed seeds of different genotypes. For each genotype the total number of differential expressed genes is indicated between brackets. In the intersection of all genotypes the number of genes that are investigated in this study are presented, 45 and 25 for the D-up and AR-up set, respectively. (c) Heat map consisting of 245 NIL*DOG1* D-up genes ($P < 0.0001$). The significance of these genes in the other genotypes is indicated, the white color indicates the genes that are not significantly different in the other genotypes ($P < 0.01$). (d) Heat map consisting of 159 NIL*DOG1* AR-up genes ($P < 0.0001$). The significance of these genes in the other genotypes is indicated, the white color represents the genes that are not significantly different in the other genotypes ($P < 0.01$). (e) Germination behaviour of freshly harvested seeds of L*er*, *dog1* and NIL*DOG1*. (f) Box plot showing the expression of the 45 D-up genes in freshly harvested imbibed L*er*, *dog1* and NIL*DOG1* seeds (expression data taken from Dekkers *et al.* (2016b)). (g) Box plot showing the expression of the 25 D-up genes in freshly harvested imbibed L*er*, *dog1* and NIL*DOG1* seeds.

Table 1: Mutants isolated from D-up genes. The table includes information about the affected gene (according to TAIR10[a])

SALK/SAIL entry	AGI code	Knock out (#)	Encoded protein
SALK_073011C	AT2G29300	KO 1	NAD(P)-binding Rossmann-fold superfamily protein (RFSP?)
SALK_028749.55.25.x	AT2G31350	KO 2	Mitochondrial glyoxalase 2 (GLX2-5)
SALK_054451.53.45.x	AT2G33830	KO 3	Dormancy/auxin associated family protein(ATDRM2)
SALK_025507C	AT2G38800	KO 4	Plant calmodulin-binding protein-related (PCBP)
SALK_082639C	AT3G14880	KO 5	Transcription factor-related
SALK_150592C	AT5G01670	KO 6	NAD(P)-linked oxidoreductase superfamily protein
SALK_059351	AT5G64210	KO 7	Alternative oxidase2 (AOX2)
SALK_104275C	AT1G01240	KO 8	Unknown protein
SALK_110011C	AT1G05840	KO 9	Eukaryotic aspartyl protease family protein
SALK_027164C	AT1G27990	KO 10	Unknown protein
SALK_036898C	AT2G19900	KO 11	The malic enzyme1(ATNADP-ME1)
SALK_037108.56.00.x	AT1G13640	KO 12	Phosphatidylinositol 3- and 4-kinase family protein
SALK_101144	AT1G56600	KO 13	Galactinol synthase(GOLS2)
SALK_138905.29.65.x	AT2G27940	KO 14	RING/U-box superfamily protein
SALK_094895	AT3G02990	KO 15	Member of Heat Stress Transcription Factor family (HSFA1E)
SALK_025488.38.10	AT3G03310	KO 16	Lecithin:cholesterol acyltransferase 3 (LCAT3)
SALK_038352	AT3G22490	KO 17	Seed maturation protein
SALK_082777C	AT3G53410	KO 18	Paralog of ubiquitin E3 ligase (LUL2)
SALK_090239C	AT3G62090	KO 19	Phytochrome-Interacting Factors (PIF6)
SAIL_512_E03	AT4G19390	KO 20	Uncharacterised protein family
SALK_137617.43.90.x	AT5G02840	KO 21	LHY/CCA1-LIKE 1 (LCL1)
SALK_101433C	AT1G13340	KO 22	Regulator of Vps4 activity in the MVB pathway protein
SALK_025893C	AT1G20650	KO 23	Altered Seed Germination 5 (ASG5)
SALK_087702C	AT1G77450	KO 24	NAC domain-containing protein 32 (NAC032)
SALK_003223C	AT1G79440	KO 25	Succinate-semialdehyde dehydrogenase 1 (SSADH1)
SAIL_563_D10	AT1G80090	KO 26	Cystathionine beta-synthase family protein (CBSX4)

SALK_078702	AT3G50740	KO 27	UDP-glucosyl transferase 72E1 (UGT72E1)
SALK_116062C	AT3G53040	KO 28	Late embryogenesis abundant (LEA)protein
SALK_082087C	AT4G09600	KO 29	Gibberellin-regulated gene family(GASA3)
SALK_112631	AT4G20070	KO 30	Allantoate Amidohydrolase (AtAAH)
SALK_105045	AT4G25580	KO 31	CAP160 protein
SALK_043547C	AT4G36700	KO 32	RmlC-like cupins superfamily protein
SALK_135551C	AT5G65280	KO 33	GCR2-like 1 (GCL1)
SAIL_1256_F11	AT5G58650	KO 34	Plant peptide containing sulfated tyrosine 1(PSY1)

a: TAIR database website: http://www.arabidopsis.org/index.jsp.

Table 2: Mutants isolated from AR-up genes. The table includes information about the affected gene (according to TAIR10[a])

SALK entry	**AGI code**	**Knock out #**	**Encoded protein**
SALK_043889	AT4G34135	KO 35	UDP-Glucosyltransferase 73B2 (UGT73B2)
SALK_070860C	AT3G26060	KO 36	PEROXIREDOXIN Q (PRXQ)
SALK_094069C	AT3G26570	KO 37	Phosphate transporter 2;1 (PHT2;1)
SALK_091600.51.00.x	AT5G49910	KO 38	Chloroplast heat shock protein 70-2 (CPHSC70-2)
SALK_097487C	AT4G34131	KO 39	UDP-glucosyl transferase 73B3 (UGT73B3)
SALK_086616C	AT3G20210	KO 40	Delta vacuolar processing enzyme (DELTA-VPE)
SAIL_547_D05	AT4G31330	KO 41	Protein of unknown function
SALK_007230.56.00.x	AT5G13400	KO 42	Peptide transporter 5
SALK_017818.55.50.x	AT2G45180	KO 43	Lipid-transfer protein/seed storage 2S albumin superfamily protein
SALK_095678	AT1G07890	KO 44	Ascorbate peroxidase 1 (APX1)
SALK_090550.52.85.x	AT1G47128	KO 45	Responsive to dehydration 21 (RD21)
SALK_015756	AT3G45010	KO 46	Serine carboxypeptidase-like 48 (scpl48)
SALK_132995.40.05.x	AT4G34260	KO 47	Altered Xyloglucan 8 (AXY8)

a: TAIR database website: http://www.arabidopsis.org/index.jsp.

Among the identified genes there were several that had been related to seed dormancy or germination before, including *PHYTOCHROME-INTERACTING FACTOR 6* (*PIF6, KO19, AT3G62090)*) (Penfield *et al.* 2010), *GIBBERELLIN 3-OXIDASE 2* (*GA3OX2*) (Yamaguchi *et al.* 1998) and *ALTERED SEED GERMINATION 5* (*ASG5,* KO23, AT1G20650) (Bassel *et al.* 2011). In addition, we found genes encoding for late embryogenesis abundant (LEA) proteins which are known to accumulate during seed desiccation and in response to water deficit induced by drought, low temperature, or salinity (Ismail *et al.* 1999, Nylander *et al.* 2001). The identified genes cover various GO molecular function categories among which by far the largest proportion is enzyme-related, including transferase activity, kinase activity and hydrolase activity, next to nucleotide binding proteins, including transcription factors.

Isolation of T-DNA mutants for genes involved in seed dormancy and germination

To investigate whether the identified genes indeed affect dormancy and AR we have analysed their T-DNA knock-out lines for seed performance phenotypes. For most of the identified genes, T-DNA mutants could be selected from the SALK and SAIL collections (NASC, http://arabidopsis.info/), but for eight genes no T-DNA insertion mutants were available (Table S1). In all cases, homozygous lines were generated and confirmed using a PCR-based approach. For 47 genes a homozygous KO mutant could be selected. For nine genes (mostly in the AR-up set) no insertion was found in any of the plants genotyped (described as 'all wild type' in Table S1). Moreover, for two genes *FRUCTOSE-BISPHOSPHATE ALDOLASE* (*FBA2*; AT4G38970) and *DELTA-9 DESATURASE1* (*ADS1* AT1G06080) T-DNA insertions were identified, but no homozygous mutants could be selected. Likely, the mutants homozygous for these genes are lethal; therefore siliques of these lines were dissected to investigate possible seed abortion. This confirmed that homozygous mutant seeds of these lines were aborted (around a quarter) at an early stage of seed development (Figure 2). Complete genotyping information is given in Table S1.

All the homozygous T-DNA lines were grown together with wild type Columbia (Col) for phenotypic analysis. This revealed normal plant phenotypes for most of

the mutants; however, for the *NAD(P)-BINDING ROSSMANN-FOLD SUPER FAMILY PROTEIN* (*NBRSFP*; KO1, AT2G29300) and *SUCCINATE-SEMIALDEHYDE DEHYDROGENASE* (*SSADH1*, KO25, At1G79440) mutants the phenotype was dramatically altered (Figure 2). After seed harvest seeds were tested for their seed performance phenotype.

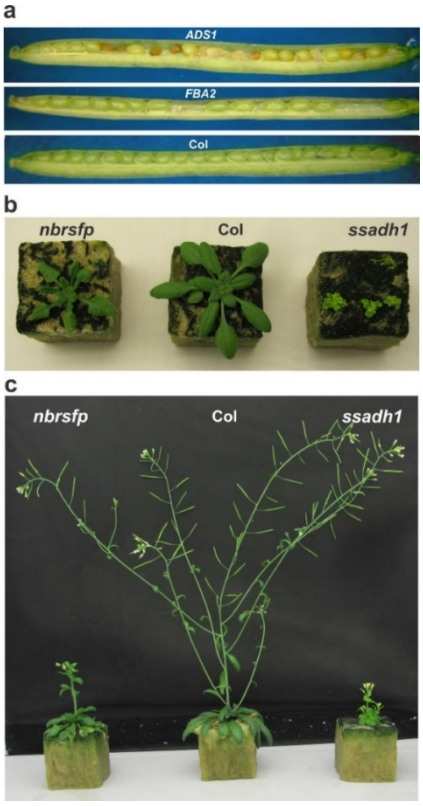

Figure 2. Plant phenotypes of T-DNA knock-out lines in comparison with wild type Columbia (Col). (a) Aborted seeds in siliques from heterozygous T-DNA lines with insertions in *FBA2* (AT1G06080) and *ADS1* (AT4G38970). (b) Four-week old plants of the NAD(P)-BINDING ROSSMANN-FOLD SUPER FAMILY PROTEIN (*nbrsfp*; KO1, AT2G29300) and *SUCCINATE-SEMIALDEHYDE DEHYDROGENASE* mutant (*ssadh1*, KO25, At1G79440) (C) *nbrsfp* and *ssadh1* six weeks after germination.

Altered seed dormancy for knock-out mutants in the dormancy and after-ripened up gene sets

Initially only seed dormancy levels were examined by assessing the number of days of seed dry storage that were required to reach 50% of germination (Days of Seed Dry Storage to reach 50% of germination; DSDS50); Figure 3a). Thereafter, the fully after-ripened seeds were tested for seed longevity (Figure 3b) and germination in salt (Figure 3c), mannitol (Figure 3d) and ABA (Figure 3e). Thirteen

lines showed a dormancy level (DSDS50) that was significantly distinct from the wild type, of which seven were less dormant (KO11, 14, 16, 17, 20, 36, 41 and 43) and six were more dormant (KO5, 16, 19, 23, 25 and 30) than the wild type. Several of the mutants were specifically affected in their seed dormancy levels, so no other seed performance phenotypes were detected. Among these mutants were a transcription factor related gene which is known to respond to karrikin (KO5, At3G14880), *LECITHIN:CHOLESTEROL ACYLTRANSFERASE 3* (*LCAT3*; KO16, At3G03310) which is involved in lipid metabolism, a seed maturation protein (KO17; AT3G22490), *SSADH1*, *PIF6*, the antioxidant gene *PEROXIREDOXIN Q* (*PRXQ*; KO36, AT3G26060), and a *SEED STORAGE 2S ALBUMIN SUPER FAMILY MEMBER* (KO43, AT2G455180). *Pif6* displayed a more than two times higher DSDS50 (27.7 days) than the wild type (11.67). *PIF6* has been previously found to negatively regulate seed dormancy (Penfield *et al*, 2010). The other mutants were affected for at least one other seed performance trait, as well. Lines with mutations in *MALATE ENZYME 1* (*ME1*, KO11, At2G19900)*, ASG5* and an *unknown protein* (*PUF*, KO41, At4G31330) displayed both a dormancy and a seed longevity phenotype. Interestingly, *atnadp-me1* and *puf* had reduced dormancy and longevity and *puf* was also less sensitive to salt stress. *Asg5* showed increased dormancy and longevity. A KO in *ALLANTOATE AMIDOHYDROLASE* (*AAH*; KO30, AT4G20070) displayed a phenotype for all investigated seeds traits except for seed longevity. This gene encodes an enzyme that hydrolyses ureide allantoate to ureidoglycolate, CO2, and two molecules of ammonium. The *aah* mutant was more dormant, and more sensitive to salt and mannitol, but less sensitive to ABA. A KO in a *U-BOX SUPER FAMILY PROTEIN* (KO14, At2G27940) appeared slightly less dormant than wild type but was far more sensitive to ABA. A KO in the *UNCHARACTERISED PROTEIN FAMILY* gene (*UPF*; At4G19390, KO20) showed a very strong non-dormant phenotype, and was rather insensitive to mannitol and salt.

Other seed performance phenotypes for D-up and AR-up genes

Among the selected mutants were also genotypes that were not affected in their seed dormancy levels but displayed altered phenotypes for other seed performance traits.

Mutants with altered seed longevity phenotype. The *nbrsfp* mutant (KO1, AT2G29300) is besides its reduced seed longevity, also more sensitive to germination in salt and mannitol. Lines mutated in *GIBBERELLIN-REGULATED GENE FAMILY* (*GASA3,* AT4G09600, KO29) and *UDP-GLUCOSYL TRANSFERASE 73B3* (*UGT73B3,* AT4G34131, KO39) showed a longevity phenotype. A role for these genes in seed longevity has not been reported previously.

Mutants with altered response to* NaCl *and/or osmotic stress. Lines mutated in mitochondrial *GLYOXALASE 2* (*GLX2*; KO2*,* AT2G31350), *DELTA VACUOLAR PROCESSING ENZYME* (*DELTA-VPE*, KO40, At3G20210) and *SERINE CARBOXYPEPTIDASE-LIKE 48* (*SCPL48*,KO46, AT3G45010) showed reduced germination in salt, but tolerated low osmotic potentials caused by high concentrations of mannitol. Although more sensitive to salt, *glx2* was more resistant to ABA than wild type. The KO in *MYB TRANSCRIPTION FACTOR LHY-CCA1-LIKE1* (*LCL1*, KO21, AT5G02840) was less sensitive to germination in mannitol compared to wild type. A similar trend was seen after germination in salt but this effect was not significant. Lines mutated in *CUPINS SUPER FAMILY PROTEIN* (KO32, AT4G36700) and *ALTERED XYLOGLUCAN 8* (AXY8; KO47, AT4G34260) showed a salt resistance phenotype but their germination in mannitol was similar to wild type. Besides lower sensitivity to salt, line *axy8* also showed reduced sensitivity to ABA.

Responses of dormancy* related *genes to ABA. Several lines that showed an altered response to ABA have already been mentioned above because they had at least also one other phenotype. However, there are three lines that showed a phenotype only for germination in the presence of ABA. Two lines, KOs in *HEAT SHOCK TRANSCRIPTION FACTOR A1E* (*HSFA1E*; KO15, AT3G02990) and *LOW AFFINITY PHOSPHATE TRANSPORTER* (*PHT2;1*, KO37,AT3G26570) were more sensitive, whereas the line mutated in *REGULATOR OF VPS4 ACTIVITY* in the MVB pathway protein (KO22, AT1G13340) was more resistant to ABA.

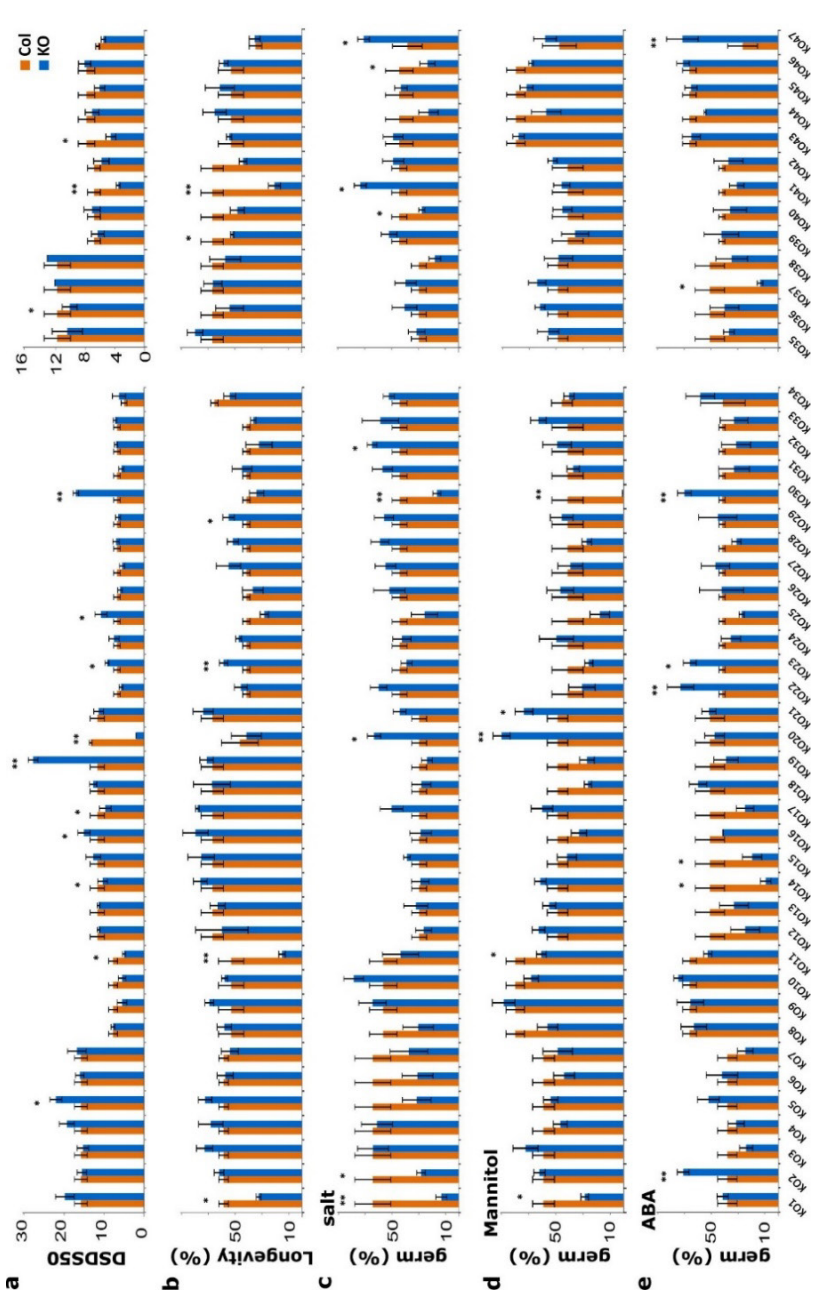

Figure 3: Germination behaviour of knock-out mutants (KO) in dormancy (left) and after –ripened (right) upregulated genes: (a) Average DSDS50 (Days of Seed Dry Storage until 50% germination) values. (b) Germination after accelerated aging. (c) Germination in salt 130mM; d) in mannitol (-1MPa) and e) in ABA (0.15 µM) solutions. Significant differences are indicated (* $P < 0.05$ and ** $P < 0.01$). There are differences in Col-0 values between the different experiments, however, every knock-out line has been compared to the Col-0 that was grown in the same experiment.

DISCUSSION

In our search for novel players in the regulation of *Arabidopsis* seed dormancy we employed a comparative transcriptomics approach for 24 hours imbibed dormant and after-ripened *DOG*NILs and L*er* seeds. The same genotypes were earlier used to investigate the transcriptome of dormant dry seeds (Bentsink *et al*, 2010), which revealed that seed dormancy in the *DOG*NILs is mainly controlled by different additive genetic and molecular pathways. In dry seeds hardly any differences are found between dormant and after-ripened seeds, however as soon as seeds are being exposed to water, differences in the transcriptomes are evident. Based on these results we hypothesize that dormancy induction in the *DOG*NILs during seed maturation, for which dry seeds are the readout, is largely regulated by distinct molecular pathways, however dormancy maintenance during seed imbibition and the start of germination are likely much conserved processes. This conservation allowed us to identify a robust set of genes which are expressed at 24 hours imbibed dormant and AR seeds. The genes identified depend a lot on the time-points chosen. From our earlier work we know that already at early imbibition (3 hours after the start of imbibition) the first differences between dormant and after-ripened seeds can be identified (Dekkers *et al*, 2016a). However, we also know that most of changes in gene expression are related to seed rehydration itself and that those changes are similar between dormant and after-ripened seeds. To exclusively identify differences that are related to dormancy maintenance and germination we have chosen to investigate the transcriptome at 24 hours after imbibition. This robustness of the identified genes was confirmed by comparison with previously published expression analyses that were performed with seeds of the Cvi accession at a range of physiological states (Cadman *et al*, 2006; Dekkers *et al*, 2016a). Of the dormancy and AR-up genes 63% and 32%, respectively, overlapped with genes that were identified by Cadman *et al*. (2006) (Table S1). Moreover, the D-up genes are also clearly higher expressed in dormant Cvi seeds as compared to AR Cvi seeds, as well as that the AR-up genes are on average higher expressed in AR seeds when compared to dormant seeds (Fig S2). Some of the identified genes had previously been shown to play a role during germination and/or priming in several plant species. Among these are *GA3OX2* (AT1G80340), a key gene in the gibberellin biosynthesis pathway, *PIF6,* involved in the phytochrome signalling pathway and

ASG5, which is involved in protein and amino acid phosphorylation. The identification of these known dormancy mutants was the incentive to investigate the other dormancy and after-ripening specific genes. We took a reverse genetics approach by using T-DNA insertion lines for the differentially expressed genes and, indeed, we identified genes that had not been related to seed dormancy or germination before. Out of our target list of 66 genes, eight do not currently have any confirmed knock-out line available. This is consistent with a recent report that 12% of Arabidopsis genes do not have insertion lines available in previously generated collections (O'Malley and Ecker 2010). The fact that a majority of the mutants showed near-wild type dormancy phenotypes, can be explained in several ways. The location of the T-DNA insertion may be decisive, e.g. whether in an intron, an exon or in untranscribed regions, such as promoters. Also, T-DNA–induced mutations do not always result in highly effective mutagenesis. Insertion in the protein-coding region of a gene generates a knockout in 86% of the cases and only 41% of the cases if the insertion is in front of the start codon (Wang 2008). Furthermore, gene redundancy may mask any phenotypic difference in plants in which the expression of only one homologue is disrupted (Hua and Meyerowitz 1998). In addition, in our experiments we used mutants with a Columbia-0 background that normally has low dormancy, which consequently does not allow the visualisation of small effects towards a decreasing dormancy level. Seed dormancy can be regulated by either inhibitory or promoting gene expression, considering the fact that in D-up genes we found both mutants that are less dormant (Figure 3; KO11, 14 and 17) and more dormant (KO5, 16, 19, 23, 25 and 30). These examples demonstrate the inability to predict phenotypes based on expression pattern alone.

Many seed performance characteristics (i.e. seed desiccation tolerance, seed longevity and seed dormancy) are acquired during seed maturation. If genes affect seed maturation in general, it is likely that pleiotropic effects occur. In our study, some of the mutants showed a phenotype for more than one germination trait. The *aah* mutant for example displayed a phenotype for all investigated seed traits, except longevity. This enzyme degrades allantoate which is required to recycle purine-ring nitrogen in plants. The *aah* T-DNA mutant is unable to grow on allantoin as sole nitrogen source (Werner *et al.* 2008). Furthermore, it is well known that

conditions favouring nitrate accumulation in mother plant may lead to lower seed dormancy levels (Alboresi *et al.* 2005). Since *AAH* is a key gene in the purine pathway (Werner *et al*, 2008), we speculate that defects in this gene block the pathway and, hence, availability of ammonium, resulting in increased primary dormancy and also affecting other seed performance traits of the mutant. *Atnadh-me1*, *asg5* and *ufp* mutants affected both dormancy and longevity and one additional trait. It is known that seed longevity can be a pleotropic effect of genes that regulate other traits, such as seed maturation (Sugliani *et al.* 2010), response to temperature (Lee *et al.* 2002), oxidative stress (Clerkx *et al.* 2004) and dormancy (Bentsink *et al.* 2006, Liu *et al.* 2007). Previous studies, using mutant analysis, have shown that the seed dormancy mutants *dog1* and *rdo4* also have a reduced seed longevity phenotype (Bentsink *et al*. 2006, Liu, *et al*. 2007).

Loss of dormancy is expressed as opening of the germination window (permissive range of environments) (Finch-Savage and Leubner-Metzger, 2006). It is because of this that germination under stress (i.e. salt or osmotic stress) often correlates with initial seed dormancy levels. We revealed two cases for which reduced dormancy indeed coincided with reduced sensitivity to salt stress (*upf* (KO20) and *puf* (KO41)). For some mutants, *nbrsfp* (KO1), *upf* (KO20) and *aah* (KO30), germination patterns on both NaCl and mannitol correlated positively, probably because both treatments confer osmotic stress. Two of these mutants (*nbrsfp* and *aah*) displayed enhanced sensitivity to salinity and osmotic stress. The NAD(P)-binding Rossmann-fold superfamily protein has oxidoreductase activity, binding, catalytic activity and, based on TAIR annotation, it is located in the endomembrane system. *Upf* was the only mutant to be clearly more insensitive to both salt and osmotic stress, which indicates that this mutant is primarily osmotolerant. Furthermore, for some of the salt-tolerant mutants *RmlC-like cupins super family protein* (KO32), *unknown protein* KO41 and *axy8* (KO47) the germination rates on mannitol were similar to that of wild type. Likely, for the salt-tolerant lines, genes were mutated whose products are elements of stress signalling and inhibit germination under conditions of saline stress. The salt sensitive *glx2* mutant was more tolerant to the application of ABA. The glyoxalase pathway consists of the two enzymes GLX1 and GLX2 and has a vital role in chemical detoxification. In *Arabidopsis thaliana,* GLX2 is required during abiotic stress, as was concluded from

the higher sensitive of *glx2-1*to salt stress and anoxia seeds compared to wild type seeds. Moreover, GLX2-1-OE seeds are more resistant to anoxic stress than wild type (Devanathan *et al*., 2014).

Interestingly, *axy8* (KO47) showed both a higher tolerance to ABA and to salt. *AXY8* encodes an α-fucosidase acting on hemicellulose xyloglucan (XyG), a process that occurs in the primary cell wall of all vascular plants. Due to its high levels in elongating tissues (Carpita and Gibeaut 1993) and structural alterations during cell elongation (Pauly *et al.* 2001), XyG has been proposed to be a major player in extension growth (Cosgrove 1999). This was confirmed by the induction of genes involved in XyG metabolism during cell elongation and upon the addition of the growth hormone auxin (Sánchez *et al.* 2003). Overall these findings emphasize the importance of cell wall remodelling in the germination process, especially in response to stress conditions.

In both the dormancy and after-ripened sets we identified many genes encoding enzymes. This result might be linked to the fact that we looked at 24-hour imbibed seeds. At this stage most of the cells in the embryo are potentially metabolically active. This also activates hydrolytic and synthetic enzymes and growth hormones to mobilize nutrients and synthesize ingredients for growth. These include the genes encoding ABA- and GA-biosynthesis- and -deactivation enzymes that play critical roles in determining the ABA-GA balance in seeds, and hence, dormancy and germination (Nambara and Marion-Poll 2005, Yamaguchi 2008).

Among the identified genes are also nucleotide binding proteins and transcription factors, such as members of the heat stress transcription factor family (*HSFA1E*, KO15), members of the ERF transcription factor family, transcription factor-related protein (KO5), TRANSCRIPTION FACTOR HOMOLOGOUS TO ABI5 and *PIF6* (KO19). Interestingly all are found in the D-up state and for the ones that mutants were analysed they showed either more dormancy (*transcription factor-related protein* and *pif6*) or more sensitivity to ABA (*hsfa1a*).

CONCLUSION

We identified seed dormancy and germination phenotypes for genes that had not been associated with seed dormancy before. We tested only one T-DNA allele per line which may not be a definitive prove that the insertional mutation causes the observed phenotype, because as many as 50% of the lines may contain additional inserts at unknown loci (Gase *et al.* 2011, O'Malley and Ecker 2010). We identified germination related phenotypes for nearly 50% of the investigated genes, which is far higher compared to what can be expected from a random selection of genes. It is also far higher compared to the genotypes that we identified for genes identified in earlier transcriptome analyses. Nevertheless, it is possible that a mutation in a second locus may cause the observed phenotype, or may alter the phenotypic effect of a knockout mutation. This work therefore represents an inventory of genes that are likely involved in the control of seed dormancy or germination. However, in depth studies are required to reveal the molecular mechanism by which these genes affect these important seed traits.

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SUPPLEMENTAL FIGURES

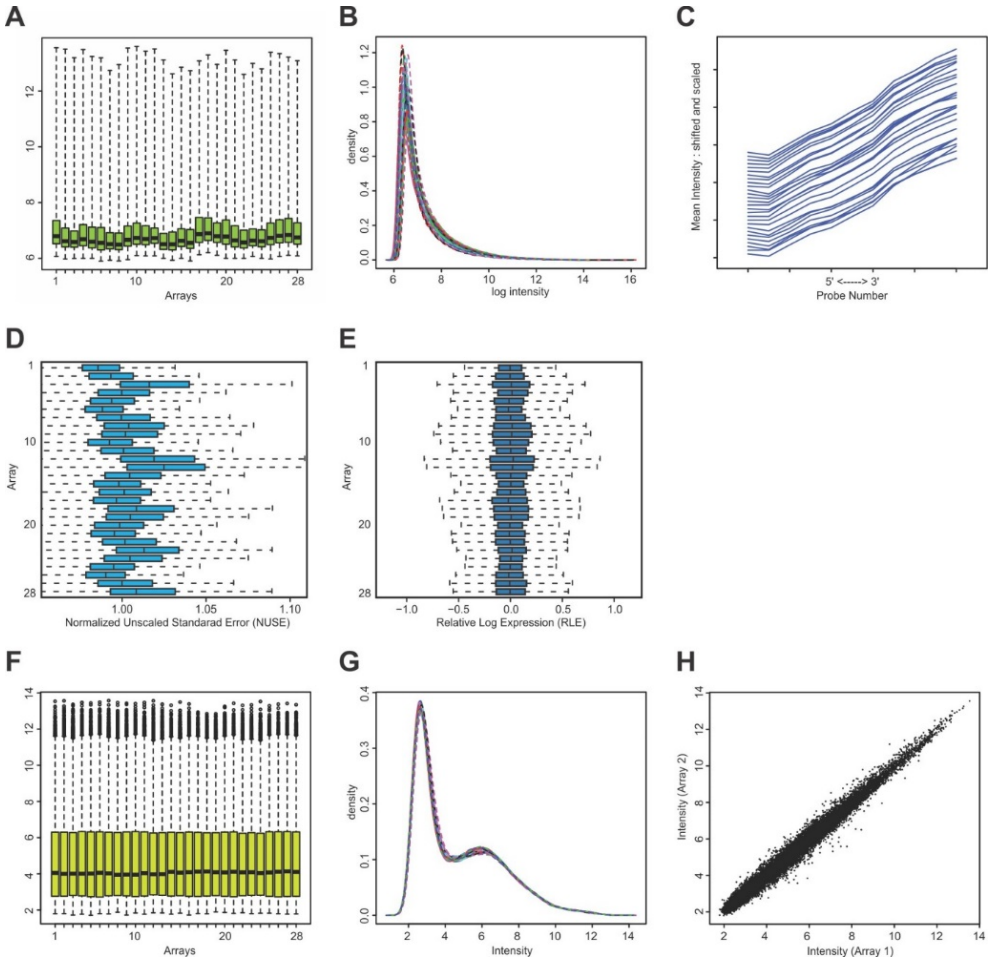

Fig. S1. Microarray quality and reproducibility. All 28 ATH1 arrays used showed after hybridization similar patterns of intensity (A and B). Slide hybridization patterns were inspected manually without detecting artefacts. The RNAs used as templates for cRNA synthesis were shown to be intact based on Bio-analyzer 2001 analysis of both RNA template and biotinylated cRNA. In agreement with this were the hybridization patterns of control genes on the slide showing a near-identical pattern of hybridization (c). The uniformity of normalized unscaled SE (NUSE) and relative log expression (RLE) indicate high quality and uniformity of the hybridization data (d and e) (1). Raw intensity data were subjected to RMA normalization (2), which kept the uniformity of general levels between the different slides (f and g). Between replicate reproducibility of the experiment was high, exemplified by the high correlation between the data of two biological replicates (h). Array 1 and array 2 are hybridized with cRNA from different replicates of L*er* seeds.

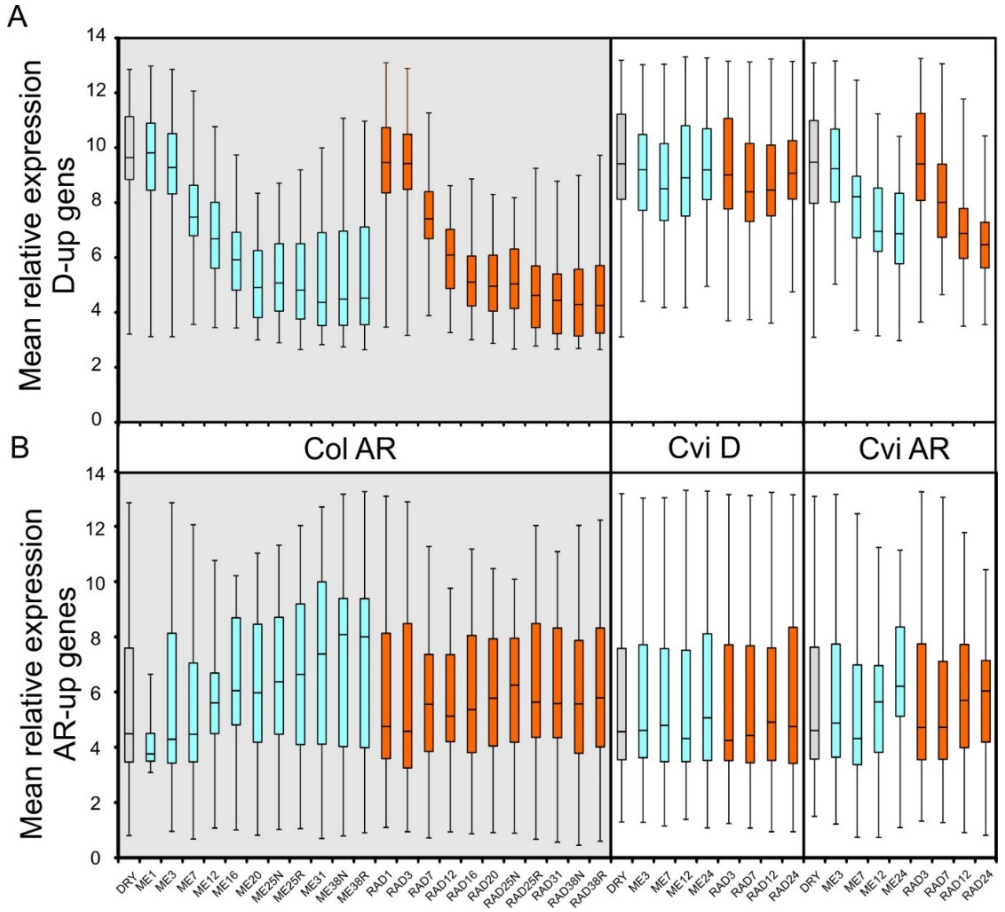

Fig. S2. Spatial and temporal expression patterns of the selected dormancy and after-ripening up-regulated genes. Mean relative expression of (a) D-up and (b) AR-up genes across the Arabidopsis germination time course in the micropylar and chalazal endosperm (MCE) and radicle and hypocotyl (RAD) in dry, 1, 3, 7, 12, 16, 20, 25, 31 and 38 hours after imbibition. Data was taken from Seed EFP Browser (http://www.bioinformatics.nl/efp/cgi-bin/efpWeb.cgi).

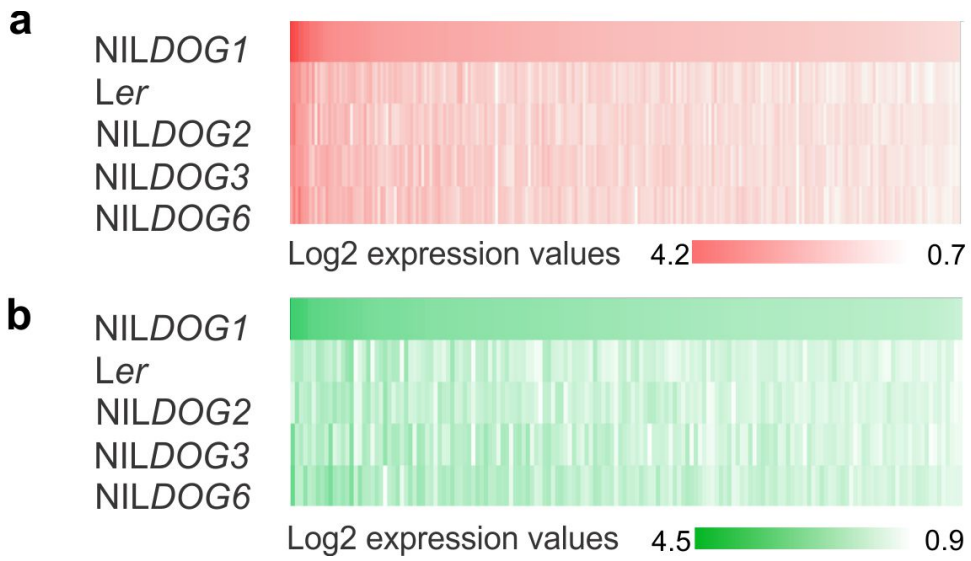

Fig. S3. Log2 expression differences for the D-up and AR-up genes that are presented in Figure 1c and d. (a) Heat map showing Log2 expression differences of the 245 NIL*DOG1* D-up genes ($P < 0.0001$) in NIL*DOG1* and the other genotypes. (b) Log2 expression differences of the 159 NIL*DOG1* AR-up genes ($P < 0.0001$) in NIL*DOG1* and the other genotypes.

Supplemental table 1: T-DNA insertion line selection of the 45 D-up (A) and 25 AR-up (B) genes. Details like, T-DNA identification, genotype, primers used for genotyping, knock-out # in the analyses, where the T-DNA is inserted and whether the genes overlap with the study of Cadman et al., 2006 are indicated.

(A)

Annotated Locus	T-DNA identifier	Genotype results	Left primer	Right primer	Knock out #	Insertion site	overlap with Cadman study
AT2G29300	SALK_073011C	homozygous	AATTTCCTTAGAACAACGGCG	TGAACCACTCACT	KO 1	promoter	YES
AT2G31350	SALK_028749.55.25.x	homozygous	TATACCTCTTAAGCGTTGTTGTCC	AAACGTCAAAGTC	KO 2	exon	YES
AT2G33830	SALK_054451.53.45.x	homozygous	AATAAGATCACCACCCAACCC	TAGGTTGATCCCT	KO 3	exon	YES
AT2G38800	SALK_025507C	homozygous	AGAAGTAGACCCACCCACCAC	CAGTTTCAGAACC	KO 4	exon	NO
AT3G14880	SALK_082639C	homozygous	AGAAGAAAGCTCGTCGGAAAC	TAGTGTTTGGGAT	KO 5	promoter	NO
AT5G01670	SALK_150592C	homozygous	GATCTCAAGCTTTCTGCATCG	TTTGACATGGAAC	KO 6	exon	YES
AT5G64210	SALK_059351	homozygous	TACCGAGTTTGGTTCGTATCG	GAGACGATATGG/	KO 7	exon	YES
AT1G01240	SALK_104275C	homozygous	CGACTAGCTGTTCTTTGCCAC	CCGACAAAACGA/	KO 8	exon	NO
AT1G05840	SALK_110011C	homozygous	TGTGCATGTGACCATTATTGG	CCCGTGTGATTTA	KO 9	exon	YES
AT1G27990	SALK_094943	homozygous	TTACCGCATGATCATCTCCTC	TGAGCCAAGAAC	KO 10	exon	NO
AT2G19900	SALK_036898C	homozygous	CACTTGCAGAGCATACCTTCC	TGCTTACCAGCAC	KO 11	exon	YES
AT1G13640	SAIL_603_A08.v1	homozygous	GATCTGAAGAAACGGAGGAGG	CTTCGAATGGTGA	KO 12	promoter	YES
AT1G56600	SALK_101144	homozygous	TCATAGACTTGATTGGTTTCCG	AACAACACAGCC1	KO 13	exon	YES
AT2G27940	SALK_138905.29.65.x	homozygous	CGACGTGGAGACATCCTAGAG	TTGGCATCCTCTT	KO 14	promoter	YES
AT3G02990	SALK_094895	homozygous	CGAACTTTGCAGGCTTATTTG	TCCATTATCTGTC	KO 15	exon	YES
AT3G03310	SALK_093100C	homozygous	TTTCCCACTCTGTTTTGATGC	ACCAGAGGCTCT(	KO 16	promoter	YES
AT3G22490	SALK_038352	homozygous	AACGTCACCGTAAGTGACTGG	AGGATGGTGTTC/	KO 17	promoter	YES
AT3G53410	SALK_082777C	homozygous	AGAAATCAATCCCTGTCCCAC	AAGATTTGGCAAA	KO 18	promoter	NO
AT3G62090	SALK_090239C	homozygous	TTCGTTTTTGGAAACGACATC	ACTCTGCGTTGAC	KO 19	exon	YES
AT4G19390	SAIL_512_E03	homozygous	CCATATGCATTCATGTCATGC	GTTACAGCCGCA(	KO 20	promoter	NO
AT5G02840	SALK_137617.43.90.x	homozygous	TGGAACATGTGCTAAAGTCCC	AATACCGGCGGA/	KO 21	intron	YES
AT1G13340	SALK_101433C	homozygous	TTCTGACATCACATCCCCTTC	TCCTCTCTTCTCT(	KO 22	exon	YES
AT1G20650	SALK_025049.21.65.x	homozygous	GATTTTTCGGAGCTTGGTTTC	ATGAGTTCGATCA	KO 23	exon	YES
AT1G77450	SALK_129854.45.75.x	homozygous	TTCCTTTCCACCAACACAAAG	GAAAGTTCCCTGC	KO 24	exon	YES
AT1G79440	SALK_003223C	homozygous	TTGAAGATCCCATAGTGCACC	AAATGGTATTGTA	KO 25	intron	NO
AT1G80090	SAIL_563_D10	homozygous	TACGCAATGCTGAGTCAGATG	TGCGAGTTAAAAC	KO 26	exon	NO
AT3G50740	SALK_078702	homozygous	CTTTAGCTGCGTGTTCCAAAC	GAGATGAGTCAG(	KO 27	exon	YES
AT3G53040	SALK_116062C	homozygous	TCAGTGAGCAAGATGCAAATG	GTCCTGAAAGCG/	KO 28	exon	YES
AT4G09600	SALK_082087C	homozygous	GCTCTCAAACACAGATTTGGC	CAAAGGAAACCAT	KO 29	promoter	NO
AT4G20070	SALK_112631	homozygous	CATGCAATGGACAATACCATG	TGGAAAGTGTTTC	KO 30	intron	YES
AT4G25580	SALK_105045	homozygous	CGATGAAAATTGCCAGAAGAG	CAGCTACGAAAC/	KO 31	exon	NO
AT4G36700	SALK_043547C	homozygous	CTTCGTACGGTTCCTCATCAG	AAAGCAAACACGT	KO 32	exon	NO
AT5G65280	SALK_135551C	homozygous	AAGATCTGCTAACTTGCGCAG	TTAGCCAAAGTAA	KO 33	exon	NO
AT5G58650	SAIL_1256_F11	homozygous	ATAAGCGTTGACGATTTGCAC	AACACGACCGAG(	KO 34	exon	YES
AT5G57220	SALK_123882C	all wild type	ACTAACATCCACCTTGCATCG	CACTGTTGTCATT	WT		NO
AT4G13180	SALK_076072C	all wild type	TGTCGTCTCGGATAAAGATGG	TCTCGCAACTTCC	WT		NO
AT4G33980	SALK_059449C	all wild type	TTATTAGCGACCCAGATGACG	GTTTCTCATTCGT	WT		YES
AT1G21410		No KO available					YES
AT2G19320		No KO available					NO
AT2G29990		No KO available					NO
AT3G14595		No KO available					YES
AT4G19110;AT4G19112		No KO available					NO
AT3G26770		No KO available					YES
AT2G41070		not analysed-Dup					YES
AT5G18450		not analysed-Dup					YES
AT5G09640		not analysed-Dup					NO

(B)

Annotated Locus	T-DNA identifier	Genotype results	Left primer	Right primer	Knock out #	Insertion site	overlap with
AT4G34135	SALK_040058C	homozygous	TAAAAGTCCAAAAGGCGAATG	AACATGTCGGCG/	KO 35	exon	NO
AT3G26060	SALK_070860C	homozygous	GAGTTCATATGCCGTGTCAGC	CTCCTCCAAACAC	KO 36	promoter	YES
AT3G26570	SALK_139411C	homozygous	GATGCATATCGACCAAATTGG	CTGAAGAAAGGG(	KO 37	promoter	NO
AT5G49910	SAIL_272_D05	homozygous	AGCAGTCCCAACATCTCACAC	ATGCGATTGACAC	KO 38	exon	NO
AT4G34131	SALK_097487C	homozygous	GATATGCCACGCTCTCTTCAG	CCGTAAATGGCA/	KO 39	exon	NO
AT3G20210	SALK_086616C	homozygous	TCAGAAGCTGGTCACAAAAGTC	TCGCGGTTTCGT/	KO 40	exon	NO
AT4G31330	SAIL_547_D05	homozygous	TGAAGGAGGATGATAAAGTTTAAGTG	TCTTCACTGCATA	KO 41	promoter	NO
AT5G13400	SALK_007230.56.00.x	homozygous	CGATGACTTTCTCTGTGAGCC	CGAAATACGCCAT	KO 42	promoter	NO
AT2G45180	SALK_017818.55.50.x	homozygous	ATGAGCACGAATAGAAGAGCG	ATCTTGTCCAGTT	KO 43	exon	NO
AT1G07890	SALK_095678	homozygous	GCAGATTCGGTTCAATTCAAG	CTTTGGTCGAGA/	KO 44	exon	NO
AT1G47128	SALK_090550.52.85.x	homozygous	ATACACGAAACCCAACAGCTG	GAAAGCAGTTGC1	KO 45	exon	NO
AT3G45010	SALK_015756	homozygous	CAGTACAGCGGTCTACGAAGC	CAATGAGGCTGA/	KO 46	exon	YES
AT4G34260	SALK_150930.53.10.x	homozygous	ACCAAATGTCAGCTTCAATGG	TCAGCGTAATATA	KO 47	promoter	YES
AT1G06080	SALK_073508.56.00.x	Aborted	TTTCGAGCTCTAACGTTTTCG	TTTGTGTGTGGAT	Aborted	exon	NO
AT4G38970	SALK_000898	Aborted	TCCATCCAACAAGATCTCTGG	TGTTCTGTTTTGC	Aborted	exon	NO
AT4G08950	SALK_098601.44.60.x	all wild type	GATCATTCGTTAGCTGGCAAC	TACCATAAACGCC	WT		NO
AT4G26790	SALK_005657	all wild type	AAATTTTGCACACGTTTCAGG	GAGTGCTTGGTG(	WT		YES
AT3G21055	SALK_075958	all wild type	TTGAATCAGTAGCGGCAGATC	TAGGGTTCCTACC	WT		NO
AT1G04520	SALK_118025.54.35.x	all wild type	AGCTATGTGGCAAAACCATTG	TTCCGTTAAGACT	WT		YES
AT1G10960	SALK_024671	all wild type	AAAACGGTTTTAGAGAAGCGC	AACTCTTGAGCG(	WT		YES
AT2G24270	SALK_021831.55.75.x	all wild type	TGCAAACCATATAAACCGGAC	TCTTCTCTCCGAT	WT		NO
AT1G03630		not analysed-ARup					NO
AT2G41420		not analysed-ARup					NO
AT3G23880		not analysed-ARup					NO
AT1G80340		not analysed-ARup					NO

Chapter 3— *FAST GERMINATING SEEDS*, a novel gene affecting seed germination in *Arabidopsis thaliana*

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ABSTRACT

Seed dormancy gives plants ecological advantages whereas efficient seed germination is important for agriculture. Successful germination and seedling establishment rely upon rapid and uniform emergence and subsequent root growth. To attain this, farmers and seed companies have used seed priming treatments for decades. Priming is an important tool to improve seed performance, but its influence on crop production and yield has not been always confirmed. Moreover, in many species priming negatively affects seed longevity. To overcome the negative effect of priming treatments, seed companies are in search of genes that affect the rate and uniformity of germination. Recently, we found that seeds of *Arabidopsis thaliana* lacking a functional *FAST GERMINATING SEEDS* (*FGS*) gene have reduced seed dormancy, faster and more uniform germination and less sensitivity to abiotic stress, while seed longevity was not affected. The expression of *FGS* mRNA is induced during seed maturation and decreases upon seed imbibition. Freshly harvested dry seeds of *fgs* show a reduced abundance of seed storage related proteins, while germinating seeds of *fgs* revealed quick release of amino acids. Furthermore, our results showed that *FGS* controls seed germination through the maternal tissue. These findings, together with localization of *FGS* in protein storage vacuoles, suggest a role for *FGS* in seed storage protein mobilization.

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INTRODUCTION

Farmers and seed companies have a strong interest to produce seeds that germinate quickly and uniformly. However, many species have developed physiological characteristics such as seed dormancy that frustrate this interest. Seed dormancy is a condition that prevents germination, even when the environment is favourable. This is particularly important during (temporarily) favourable conditions and also for avoiding pre-harvest sprouting. Despite the ecological advantages of dormancy, it is an unwanted trait for crops that require rapid and uniform germination after sowing. To overcome the problems of slow and heterogeneous germination and seed dormancy, several techniques have been developed by the seed industry, of which seed priming is the most widely used. Seed priming is a pre-treatment of seeds for which several methods are available, including hydropriming and osmotic priming (Hussain et al., 2006; Paparella et al., 2015). A drawback of improving germination rate and uniformity by priming is the fact that it negatively affects seed longevity (Chiu et al., 2002; Hill et al., 2007). Therefore, attempts to find genes that affect germination rate and uniformity without reducing seed longevity are essential to seed producers.

Seed germination characteristics are shaped during seed development. Seed development can be divided into three phases: in the first phase, known as early embryo morphogenesis, the embryo develops through a series of cell divisions and differentiation. The second, or maturation phase, is dominated by storage compound accumulation, accounting for approximately 40% of the seed's dry matter at the end of this stage. In the final phase, the seed enters a quiescent state at which dormancy, longevity and desiccation tolerance develop (Baud et al., 2002). Seed dormancy and germination are complex traits influenced by both the genetics and the environment. In the model plant *Arabidopsis thaliana*, seeds are primary dormant upon shedding from the mother plant. They require a cold treatment (stratification) or dry after-ripening to become non-dormant (Bewley, 1997; Bewley et al., 2013). Seed germination begins with water uptake by the dry seed (imbibition) and is completed when the radicle has protruded through the covering layers (germination *sensu stricto*) (Bewley and Black, 1994). The protrusion

coincides with post-germination events when stored reserves are being mobilized to support seedling growth (Bewley, 1997).

Seed storage proteins (SSPs) are the major proteins that are rapidly hydrolysed upon germination to generate a source of nitrogen and carbon (Higgins, 1984; Duranti et al., 2008). *Arabidopsis* seeds contain two types of storage proteins, cruciferins and napins. Cruciferins are 12S globulins encoded by four paralogous genes namely *CRUA*, *CRUB*, *CRUC* and *CUPIN* (Job et al., 2005). Napins are five 2S albumins referred to as *At2S1* to *At2S5* (van der Klei et al., 1993). Both 12S globulins and 2S albumins are initially synthesized as a precursor at the endoplasmic reticulum (ER), their mature polypeptides accumulate and are densely packed inside specialized vacuoles, called protein storage vacuoles (PSVs). The PSVs are surrounded by a tonoplast and occupy most of the cellular volume by seed maturity. The membranes, or tonoplast, of PSVs contain active transporters for ATP and pyrophosphate-driven proton transport (Müntz, 1998; Jauh et al., 1999; Robinson and Hinz, 1999; Jiang et al., 2000; Holkeri and Vitale, 2001).

Here we present the physiological analysis of *FAST GERMINATING SEEDS* (*FGS*). *FGS* (At4g19390) was first identified as a dormancy-up gene, based on its higher expression in 24-hours imbibed dormant seeds as compared to after-ripened seeds (Yazdanpanah et al., 2017). The *fgs* knock-out mutant seeds display reduced seed dormancy and the seeds germinate much faster and more uniform than wild type Columbia-0 (Col-0). In the present study, we further investigated the role of *FGS* in seed germination, using metabolomics, proteomics and genetic analysis. Our results support the involvement of *FGS* in protein reserve mobilization in seed.

MATERIALS AND METHODS

Plant material

Homozygous T-DNA insertion mutant *fgs* (SAIL_512_E03; At4g19390) was isolated by a PCR-based reverse genetic screen for T-DNA insertions in the corresponding gene. Reciprocal F1 seeds of a cross between Col-0 and *fgs* mutant were generated for genetic analyses.

Generation of plant transformation vectors and transgenic Arabidopsis

SESA3:GFP:FGS constructs were generated by using the MultiSite Gateway (Three-Fragment Vector Construction kit (Life Technologies)). A seed specific promoter (SESA3; cloned from pAlligator2) was introduced in the pDONR P4-P1R; green fluorescent protein (*GFP*; cloned from pK7FWG2.gb) was introduced in the pDONR 207 P1-P2 and *FGS* gene amplified from genomic DNA of *Arabidopsis thaliana* was introduced in the pDONR P2-P3R. All three fragments were combined in an LR reaction into the pK7m34GW vector. This GFP fusion construct was transformed into Col-0 plants with *Agrobacterium tumefaciens* (GV3101; Koncz and Schell, 1986) using the floral dip method (Clough and Bent, 1998). Transgenic lines were selected on 30 μg ml^{-1} kanamycin plates. The same construct was introduced into the *fgs* mutant for mutant complementation. Transgenic plants were selected on medium containing hygromycin (50 μg ml^{-1}). The sequences of the primers used to amplify the segments are presented in Supplemental table 2.

Germination Assays

Germination experiments were performed as described by Joosen et al. (2010) with small adjustments. In brief, two layers of blue germination paper were equilibrated with 48 ml demineralized water in plastic trays (15 × 21 cm). 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 twice a day for a period of 7 days using a Nikon D80 camera fixed to a repro stand with a 60 mm macro objective. The camera was connected to a computer with Nikon Camera Control Pro software version 2.0. Percentages germinated seeds and rate of germination (T50) were calculated.

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 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. Stress conditions were: osmotic stress (-1 MPa mannitol; Sigma-Aldrich), salt stress

(130 mM NaCl; Sigma-Aldrich), ABA stress (0.15 µM 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 ZnSO4 solution (40°C, 85% relative humidity) in a closed tank for 2 or 5 days (Powell and Matthews, 2012). The most discriminative incubation time was presented. After incubation the seeds were taken out and germinated on demineralized water as described before.

***FGS* expression**

RNA was isolated using the Nucleospin RNA plant kit (Macherey-Nagel: 740949) according to the manufacturer's protocol with minor modifications: 3-5 mg freshly harvested seeds were used for the extraction. Lysis was performed using 315 µL buffer RAP + 35µL Plant RNA Isolation Aid (Ambion: AM9690) + 3.5µL β-Mercaptoethanol (Sigma: M6250). Final RNA was eluted in 40µL RNAse free water. Quality and concentrations were measured by loading 2µL 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 750ng RNA using the iScript cDNA Synthesis Kit (Bio-Rad: 170-8890) according to the manufacturer's protocol. cDNA was diluted 10 times with sterile milliQ water. For each sample 2.5µL cDNA, 5µL iQ SYBR green supermix (Bio-Rad: 172-5125) and 0.5µL primer mix (10µL work solution) were added and supplemented with water to 10µL. RT-qPCR was performed on a CFX connect (Bio-Rad).

Primer pairs used were as follows: FGS forw: 5´ GGGGATTGAGAAGGTCAT -3´ and FGS rev: 5´ CGAGCAAAGATCCTAATGTT-3´. *FGS* expression was normalized by the expression of two reference genes that are stably expressed in dry seeds: At4g12590 and At4g34270 (Dekkers et al., 2012). 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

Metabolite extraction was performed on dry mature seeds of the *fgs* mutant and Col-0 wild type 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 five mg of seeds pre-cooled in liquid nitrogen, was homogenized in 2 ml tubes with 2 iron balls (2.5 mm) using a micro dismembrator (Mo Bio Laboratory). 233 µl methanol/chloroform (4:3) was added, together with 50 µl standard (0.13 mg ml-1 ribitol) and mixed thoroughly. After 10 minutes of sonication 66 µl MQ water was added to the mixture followed by vortexing and centrifugation (5 min., 15000 rpm). The methanol phase was collected in a glass vial. 166 µl methanol/ chloroform (1:1) was added to the remaining organic phase and kept on ice for 10 min. 66 µl MQ water was added followed by vortexing and centrifugation (5 min., 15000 rpm). Again the methanol phase was collected and mixed with previously collected phase. 60 µl 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 analysed by a GC-TOF-MS system consisting of an Optic 3 high-performance injector (ATAS) and and Agilent 6890 gas chromatograph (Agilent Technologies) coupled to a Pegasus III time-of-flight mass spectrometer (Leco Instruments). 2 µl of each sample was introduced to the injector. The GC-TOF-MS method was implemented as described by (Carreno-Quintero et al., 2012) with some minor modifications. Detector voltage was set at 1650V.

Amino acid measurement

Freshly harvested Col and *fgs* seeds (5 – 10 mg) that were stored at - 80°C were sown according to the methods described for the germination experiments. Stratified seeds were incubated at 4°C for 70 hours, freshly harvested seeds were immediately germinated in an incubator at 22°C. Seeds were collected at time points of 10% and 25% germination for both freshly harvested non-stratified and stratified seeds. Seeds were crushed with liquid nitrogen (1 min, 30 Hz) and amino acids were extracted by adding 500 µl extraction buffer (80% EtOH and 10 pmol/µl isotopically labelled d3-Ala as internal standard). After vortexing for 1 min and 10 minutes of sonication, samples were centrifuged for 5 min at 15,000 x g. Of the supernatant 250 µl was transferred to a new tube and 500 µL of 80% EtOH was added to the pellet. Again, this was vortexed, sonicated and centrifuged and 250 µl supernatant was mixed with the previously obtained supernatant (end concentration

of d3-Ala is 5 pmol). This was filtered with a 0.45 µm filter (PHENOMENEX) and syringe. Derivatization was performed according to the manual of the AccQ agTM Ultra Derivatization kit (Waters), by adding 20 µl of 6-aminoquinolyl-N-hydroxysuccinidyl carbamate to 10 µl of filtered sample in 70 µl borate buffer. After vortexing, samples were incubated at room temperature for 1 min and the derivatization process was ended by heating to 55°C for 10 min. A calibration curve was prepared by mixing equimolar amounts of d3-Ala and amino acid hydrolysate standard (provided with the kit) at concentrations ranging from 3.9 pmol/µl to 500 pmol/µl.

2D gel electrophoresis

Protein separation was performed with 20 µl of protein extract, equivalent to about 150 µg of total protein. 2D gel electrophoresis was conducted as described before (Rajjou et al., 2011), adapted for gel strips forming an immobilized nonlinear pH gradient from 3 to 11 (Immobilized DryStrip pH 3-11 NL, 24 cm; GE Healthcare). For each analysed sample, 2D gels were made with at least three biological replicates. 2D gels were stained with silver nitrate. Silver-stained gels were placed between two layers of cellophane membrane stretched on cassette frames for drying. Images of dry gels were scanned with a Sharp JX-330 scanner (Arc et al., 2012). Quantitative image analysis was carried out with Progenesis Samespot software (v3.2, NonLinear Dynamics) to detect kinetics of protein accumulation and protein comparison of different samples. The SameSpot software was used to perform a one-way ANOVA for each spot. Spot abundance changes with fold change higher than 1.5 (up or down) and a p-value ≤ 0.05 in the comparison between all the established groups of biological replicates were retrieved.

Protein identification

From the spots of interest, proteins were isolated from 2D gels, digested with trypsin and identified by LC-MS/MS (Supplemental Table 1)

Confocal Microscopy

Localization studies of the GFP-FGS fusion protein were carried out using a confocal laser-scanning microscope on tissue sections of *A. thaliana* seeds (17 day after flowering) after removing their seed coats. Sections were transferred to

microscope slides, embedded in glycerol: water (1:1; v/v). The settings for collecting GFP signals were done according to the manufacturer's instructions.

RESULTS

Expression of *FAST GERMINATING SEEDS* (*FGS*) is reduced by seed imbibition

Analysis of *FGS* expression in wild type Col-0 revealed that it is mainly expressed in seeds and stem tissues. *FGS* transcripts are first detectable at three days after pollination and peak during late seed maturation reaching the highest abundance in freshly harvested dry seeds (Figure 1). Transcript abundance was reduced by after-ripening, and upon seed imbibition the expression decreased further, which was already detectable after 6 hours of imbibition in both freshly harvested and after-ripened seeds.

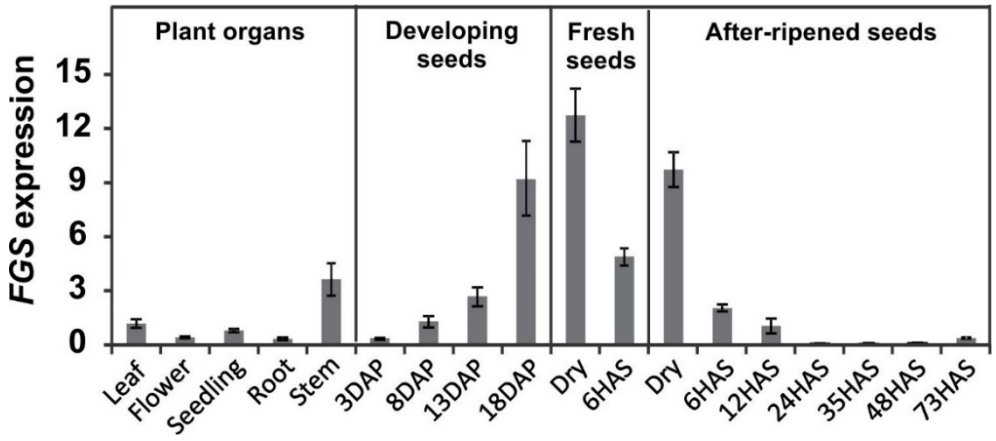

Figure 1. Relative expression of *FGS*. RT-qPCR results for *FGS* during plant development in Col-0 wild type; leaves, flowers, seedlings, roots, stems, and siliques at 3, 8, 13 and 18 days after pollination (DAP); in freshly harvested seeds at 0 (Dry) and 6 hours after sowing (HAS); in after-ripened seeds during the seed imbibition time course at 0 (Dry) 6, 12, 24, 35, 48 and 73 HAS. Shown are averages of 3-4 biological replicates and their SE.

Phenotypic analyses of the *fgs* mutant

Seeds from the *fgs* mutant were analysed for several germination parameters. Compared to wild type Col-0, seeds of the *fgs* mutant were less dormant (Fig 2A), had a lower T50 (time to reach 50 % germination; Fig 2B), were less sensitive to

germination in salt and the non-ionic solute mannitol, but germination on abscisic acid (ABA) and after artificial aging were similar to Col-0 (Fig 2C). In addition, *fgs* plants displayed rosette leaves with a shorter petiole, and were smaller in size with thicker stems in comparison to that of wild type Col-0 (Fig 2D).

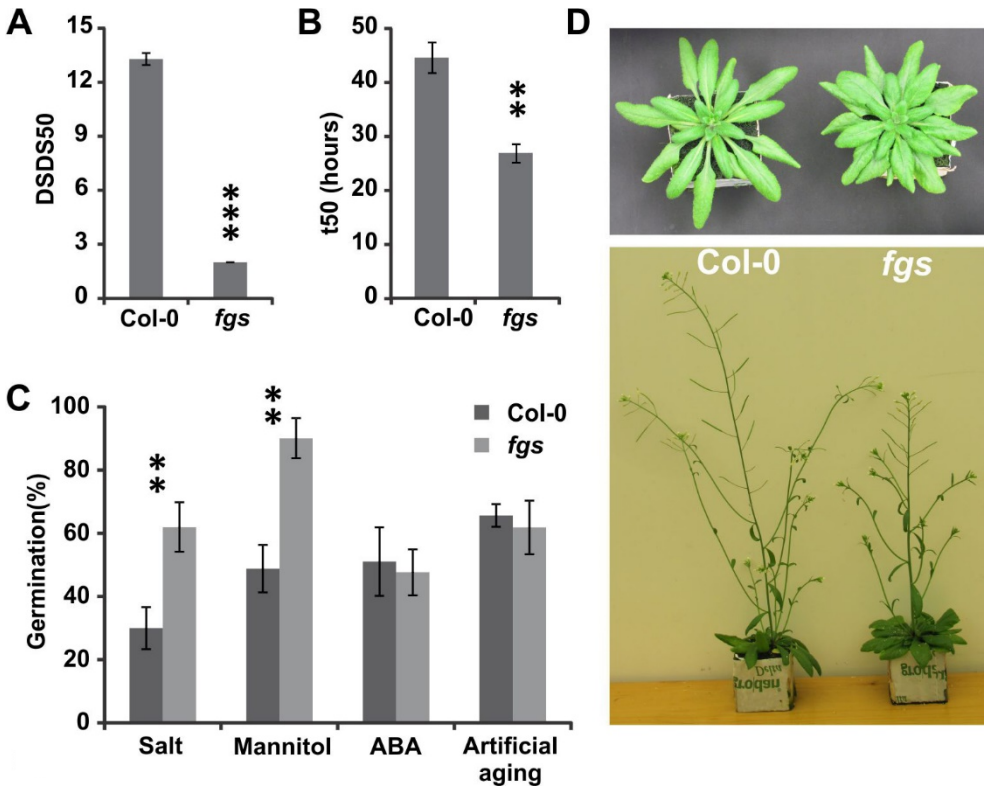

Figure 2. Phenotypes of T-DNA knock-out lines of *FGS* in comparison to wild type Col-0. A) Seed dormancy levels of Col-0 and *fgs* expressed as Days of Seed Dry Storage required to reach 50% of germination (DSDS50). B) t50 (time required for 50% of viable seeds to germinate) for Col-0 and *fgs*. C) Germination of after ripened *fgs* and Col-0 seeds in salt (130mM), mannitol (-1MPa), ABA (0.15 µM) and after artificial aging. Significant differences between Col-0 and *fgs* are indicated (three asterisks indicate $p < 0.001$; two asterisks, $p < 0.01$). These seeds germinated to 100% in water. D) Altered plant phenotype of *fgs* mutant, four (top) and six (bottom) weeks after germination.

Complementation cloning of *fgs*

Conclusive evidence that the phenotype observed in *fgs* plants is indeed due to disruption of the *FGS* gene was obtained by transforming the *fgs* mutant with a wild type copy of *FGS* expressed from the seed specific SESA3 promoter (Figure 3). The dormancy level of two independent complementation lines significantly differed from that of the *fgs* mutant. This confirmed that the observed phenotypes in the mutant were caused by disruption of the *FGS* gene and is not due to an additional T-DNA insert or spontaneous mutation (O'Malley and Ecker, 2010; Gase et al., 2011).

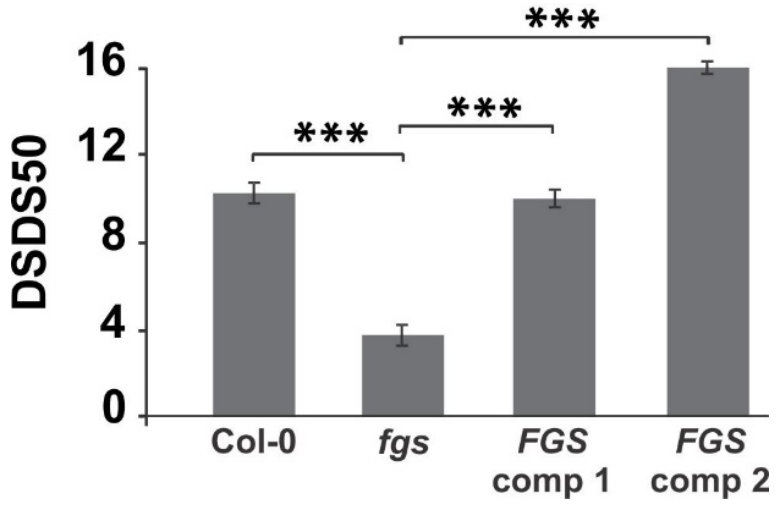

Figure 3. Genetic complementation of the *fgs* rescued the mutant phenotype. Comparison of the seed dormancy levels expressed as Days of Seed Dry Storage required to reach 50% of germination (DSDS50) of Col-0, *fgs* and two independent *fgs* complementation lines. Significant differences in comparison to *fgs* have been indicated (three asterisks, $p < 0.001$).

Metabolic changes in *fgs* loss-of-function mutant seeds

In order to get a better understanding of the function of *FGS*, we performed metabolomic analysis on freshly harvested dry and imbibed seeds of the Col-0 wild type and *fgs* mutant. In dry seeds, no significant changes were detected between the mutant and Col-0 wild type. However, in 24-hours imbibed seeds the amino acids threonine, isoleucine, glycine and serine and ascorbic acid and arabinose showed clearly higher abundance in the *fgs* mutant (Supplemental Figure 2). The

increase in amino acid contents might be explained by a difference in physiological stages of Col-0 and *fgs* seeds, due to the faster germination of *fgs.* Thus, the mutant seeds could be at a more advanced stage of germination when the seeds were collected. To investigate this more precisely, seeds amino acid contents were determined at the same physiological stages based on their germination frequency at two time points (10% and 25% germination; Figure 4CD). To investigate whether these changes are dormancy related both stratified and non-stratified seeds were analysed (Figure 4AB).

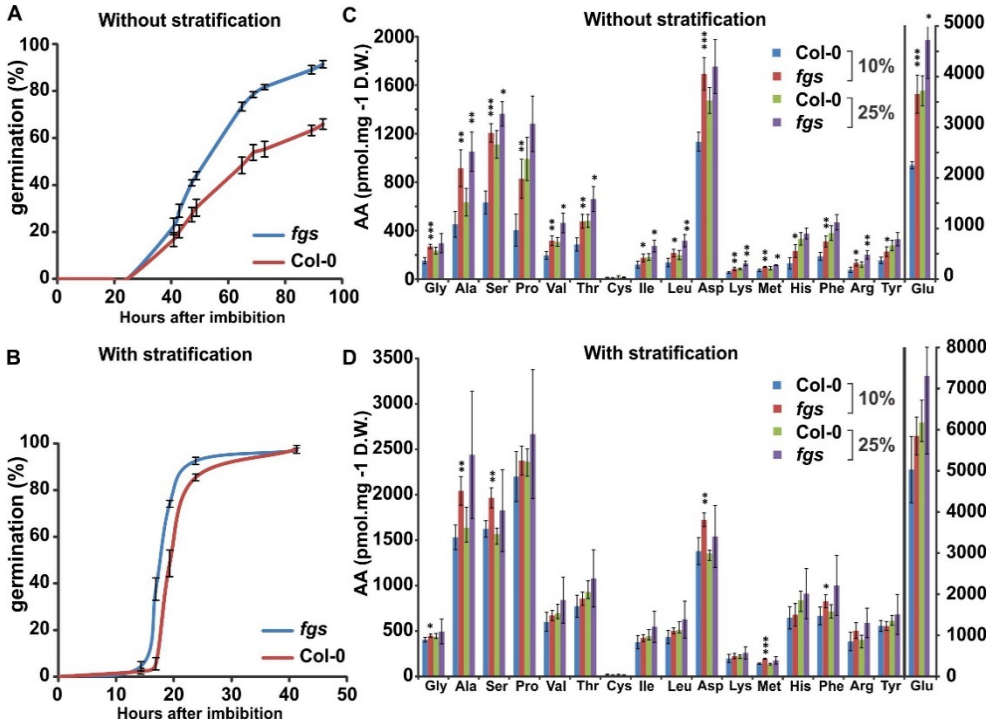

Figure 4. Higher amino acid abundance in *fgs* seeds. Germination curves of freshly harvested *fgs* and Col-0 seeds at different intervals after imbibition, A) without stratification and B) with stratification. C and D) Amino acid abundance in germinating *fgs* and Col-0 seeds. Two germination stages (10% and 25% germination) for both Col-0 and *fgs* seeds were used for AQC derivatized amino acid analysis. Relative amino acid amounts (pmol/mg D.W.) are presented. C) Without stratification and D) with stratification. Significant differences between Col-0 and *fgs* are indicated (three asterisks, $p < 0.001$; two asterisks, $p < 0.01$; one asterisk, $p < 0.05$). Note Glu levels are presented on the second Y-axis on the right.

In general, the amino acid content for the individual genotypes increased from 10% to 25% germination, especially for un-stratified seeds. Comparing *fgs* and Col-0 seeds at the same physiological stage, all amino acids at 10% germination and most of these at 25% germination in un-stratified seeds showed higher abundance in the mutant. In the case of alanine and aspartate the abundance of these amino acids in the mutant at 10% germination was even higher than Col-0 at 25% germination. For the stratified seeds only the amino acids glycine, alanine, serine, aspartate, methionine and phenylalanine at 10% germination showed higher abundance in *fgs* as compared to Col-0 and at 25% germination there was no difference.

A role for *FGS* in reserve mobilisation

FGS is highly expressed during seed maturation and in the dry state when storage proteins are protected against degradation. However, its expression is reduced upon imbibition and during germination when seeds storage proteins start to be degraded. The degradation of storage proteins upon seed imbibition is supported by an increase in amino acid levels. As shown, this increase is faster in the *fgs* mutant when compared to Col-0. To gain a better understanding of the influence of the *fgs* mutation on the protein level, the proteomes of freshly harvested dry Col-0 wild-type and *fgs* seeds were characterized (Figure 5A). Already in the one-dimensional polyacrylamide gel electrophoresis (1D PAGE) differences between Col-0 and *fgs* dry seed were observed, which was an incentive to perform 2D PAGE analysis (Figure 5B). This analysis revealed 45 protein spots, the abundance of which was increased (18) or decreased (27) in the freshly harvested dry *fgs* seeds, as compared to wild type Col-0 (e.g., Figure 5C; Supplemental table 1). Among these were many seed storage related proteins, including all 12S globulins cruciferins (CRUA, CRUB, CRUC and *CUPIN*) which displayed lower abundance in *fgs*. Instead the enzymes related to storage protein catabolism and amino acid biosynthesis were more abundant in *fgs* seeds (Supplemental table 1).

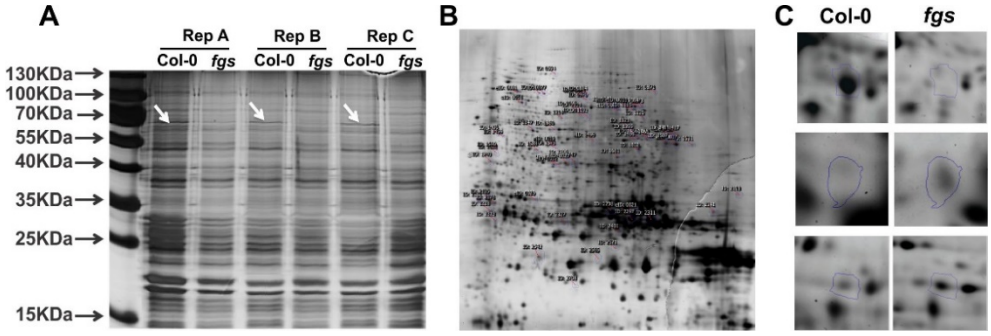

Figure 5. Characterization of *Arabidopsis* proteins with differential abundance in freshly harvested dry seeds of Col-0 and the *fgs* mutant. A) One-dimensional gel electrophoresis stained with Coomassie Brilliant Blue of total seed protein extracts of Col-0 and the *fgs* mutant. B) Silver-stained two-dimensional gel of total proteins from freshly harvested dry seeds of the *fgs* mutant. C) Enlarged windows of two-dimensional gels comparing the differential abundance of polypeptides in Col-0 to that of *fgs* mutant seeds. Equal amounts of total protein extracts were loaded onto the gels.

The localisation of the FGS protein is in accordance with its effect on protein accumulation. N-terminal green fluorescent protein (GFP) fusion proteins of *FGS* under a seed specific promoter (SESA3; At4g27160) revealed that FGS is localized in protein storage vacuoles (PSV) (Figure 6).

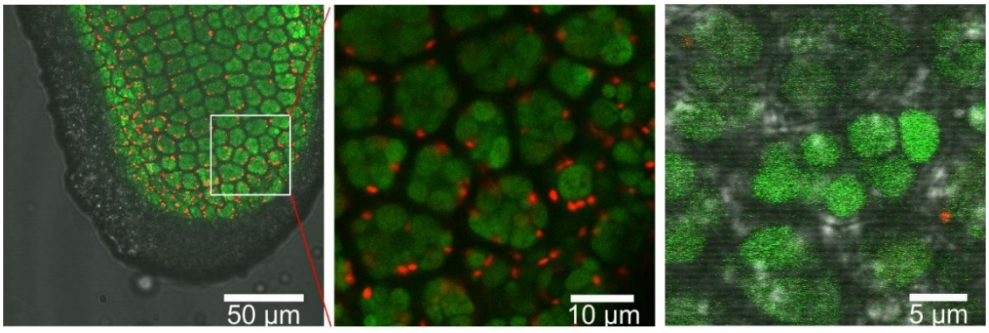

Figure 6. The FGS protein is targeted to protein storage vacuoles in Arabidopsis embryo cells. Subcellular localization of GFP:FGS in the radicle tip of embryos, in seeds harvested at 17 day after flowering. Different magnification are presented, scale bars are indicated.

FGS controls seed germination through the maternal tissue

To determine how *fgs* is genetically controlled, reciprocal crosses were made between Col-0 wild-type and *fgs*. Crosses between wild-type ovules and pollen from the mutant line produced seeds with germination phenotype of the wild-type (Figure 7). While crosses between ovules from the mutants with wild-type pollen produced seeds with germination phenotype of the mutant (Figure 7). The dependence of the germination phenotypes on maternal origin suggests that FGS controls seed germination through the maternal tissue.

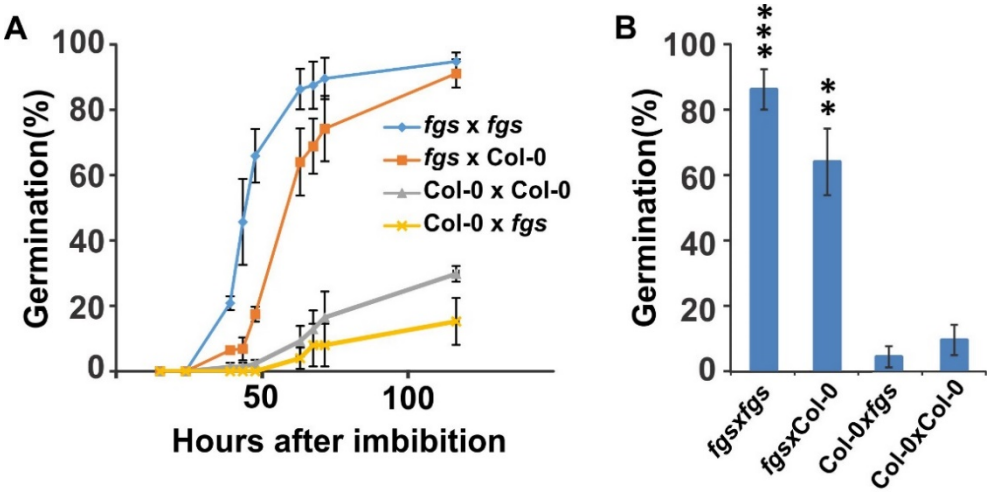

Figure 7. *FGS* maternally controls seed germination. A) Germination of Col-0 and *fgs* mutant seeds and their reciprocal F1 hybrids at different times after imbibition. B) Germination percentages of the genotypes presented in A after 63 hours of imbibition, significant differences with Col-0 × Col-0 are indicated (three asterisks, $p < 0.001$ and two asterisks, $p < 0.01$).

DISCUSSION

In the present study we performed a physiological analysis of *FAST GERMINATING SEEDS*, a novel gene whose lack of function results in seeds with reduced dormancy, faster and more synchronized germination and reduced sensitivity to abiotic stresses, without affecting seed storability.

Based on our results we propose that *FGS* affects germination via the seed maturation phase. During maturation seeds accumulate storage reserves and become desiccation-tolerant and dormant. The expression of *FGS* mRNA is enhanced during seed maturation. Other features that point towards an involvement of *FGS* in seed maturation and specially protein reserve mobilization are the differential abundance of seed storage proteins in freshly harvested *fgs* seeds and a quick release of amino acids in germinating *fgs* seeds. Furthermore, the effect of the *FGS* disruption on seed germination proceeds through the maternal tissues. A maternal effect on germination usually is through the maternal tissues surrounding the embryo, as is for example the case for the *transparent testa* mutants (Roach and Wulff, 1987; Debeaujon et al., 2000).

Seed storage proteins (SSPs; cruciferin A, B, C and CUPIN) abundance decreased in mature seeds of the mutant. This can either be due to less accumulation of storage proteins during seed maturation, or due to early SSP degradation at the end of seed maturation, or by both. This is supported by the higher levels of enzymes in *fgs* seeds that are related to storage protein catabolism and amino acid biosynthesis (Supplemental table 1), including peptidase M1 family protein (function in metallopeptidase activity with broad substrate specificity for several peptides), SBT1.7 (subtilisin-like protease) and PGDH1 (3-phosphoglycerate dehydrogenase, involved in l-Ser biosynthesis). Moreover, VHA-A (catalytic subunit A of the vacuolar H^+-ATPase) that has proton pump activity was higher in the mutant. In contrast RPT5a (one of the six AAA-ATPases of the proteasome regulatory particle) that has specific functions through the regulation of protein degradation displayed higher abundance in Col-0. RPT5a is highly expressed in dormant seeds and mutating this gene leads to reduced dormancy (Hayashi and Hirayama, 2016).

Although most cruciferins have a higher abundance in Col-0, cruciferin A1 (CRA1) isoforms were identified both at higher and lower abundance in the *fgs* mutant.

Likely, modifications of the protein causes a shift in the gel, which is identified as higher or lower abundance. It has been shown that the four cruciferin genes generate more than 100 protein isoforms because of the high sensitivity of SSPs to post-translational modifications (Arc et al., 2011). Since we did not identify all these isoforms we cannot draw conclusions on the absolute amounts of these CRA1 isoforms; however, the total protein content was equivalent in the *fgs* and Col-0 seeds.

The lower sensitivity to both mannitol and salt stress are other remarkable germination characteristics of after-ripened *fgs* mutant seeds. It is known that negative effects of salt on plants are a consequence of both insufficient water absorption and excessive Na^+ concentrations in the cytoplasm. Plants developed two common mechanisms to deal with this problem: (1) The accumulation of compatible solutes such as mannitol, sorbitol and amino acids; the accumulation of amino acids in the *fgs* mutant seeds might thus be the result of, or reason for, the mannitol tolerance; (2) Reducing sodium ions by antiporters located in the plasma membrane and tonoplast. The *fgs* mutant also showed more tolerance to salt stress suggesting a role for *FGS* in inhibiting the proton pumps located in the PSV tonoplast.

Amino acid abundance was higher in the *fgs* mutant of non-stratified seeds, but after stratification the number of amino acids with higher abundance in the mutant was smaller and for those amino acids differences between the mutant and Col-0 were less significant compared to non-stratified seeds. This might be explained by the fact that stratified seeds had already been imbibed for four days before the seeds were transferred to conditions that allowed germination. This is in line with the *fgs* phenotype that resembles primed seeds in the sense that they have a reduced dormancy and a quick and uniform germination. Stratification promotes the germination (and with that storage reserve mobilisation) of wild type seeds and therefore reduces the difference between mutant and wild type.

CONCLUSION

Loss-of-function of *FGS* reduces seed dormancy and leads to a rapid and uniform germination. Its maternal origin and the quicker release of amino acids suggest that FGS is involved in reserve protein mobilization and negatively regulating germination. These characteristics make *FGS* an interesting candidate gene for the seed industry. Mutating *FGS* results in fast and uniform germination characteristics without the negative effects on seed longevity. Moreover, knocking out *FGS* also improves germination under osmotic stress. However, whether *fgs* mutants would generally perform better in drought conditions needs to be investigated.

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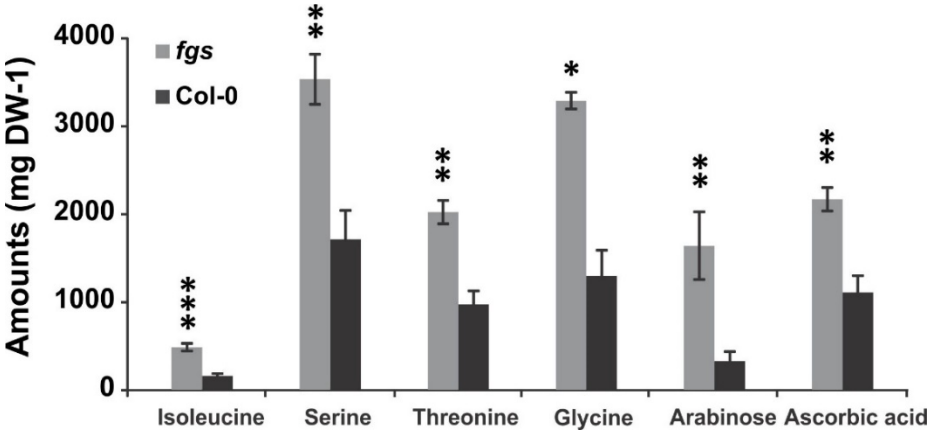
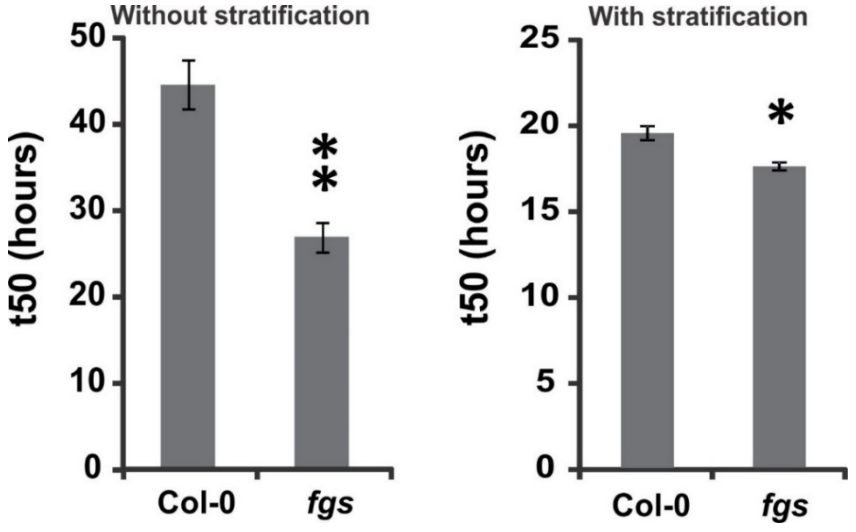
SUPPLEMENTARY INFORMATION



Supplemental Figure 1. Metabolite differences between the *fgs* mutant and Col-0 wild type. Relative metabolite amounts (mg DW^{-1}) in Col-0 and *fgs*. Metabolite levels were measured by GC-MS (see Material and Methods for details). Asterisks indicate significant differences relative to the respective wild type (three asterisks, $p< 0.001$; two asterisks, $p < 0.01$; one asterisk, $p < 0.05$).



Supplemental Figure 2. Both stratified and unstratified *fgs* mutants germinate faster than Col-0. T50 (time required for 50% of viable seeds to germinate) of *fgs* and Col-0, with and without stratification.

Supplemental table 1. *Arabidopsis* polypeptides whose abundance was significantly different in freshly harvested dry *fgs* mutant seeds compare to their dry dormant wild type, Col-0 seeds. Positive fold changes indicate higher abundances in Col-0 and negative lower abundances in Col-0.

Spot ID	Accession No	Arabidopsis Protein Name	Coverage (%)	Fold change (Col-0/*fgs*)	Experimental Molecular Mass (kD)
ID: 1459	AT3G05530.1	RPT5A, ATS6A.2 regulatory particle triple-A ATPase 5A	27.36	3.6	47.45
ID: 1347	AT2G36640.1	ATECP63, ECP63 embryonic cell protein 63	12.05	3.3	48.46
ID: 2113	AT4G05320.2.	Polyubiquitin 10	27.57	2.8	51.17
eID: 0001	AT5G28540.1	BIP1 heat shock protein 70 (Hsp 70) family protein	30.49	2.8	73.58
ID: 1429	AT1G20630.1	CAT1 catalase 1	9.96	2.8	56.73
ID: 2161	AT5G13850.1	Nascent polypeptide-associated complex subunit alpha-like protein 3	28.92	2.6	22.04
ID: 1458	AT4G28520.1	CRU3, CRC cruciferin 3	69.27	2.5	58.20
ID: 1323	AT4G05320.2	UBQ10 polyubiquitin 10	27.57	2.5	51.17
ID: 1417	AT4G28520.1	CRU3, CRC cruciferin 3	69.27	2.3	58.20
ID: 1590	AT3G13235.3	DNA damage-inducible protein 1	35.59	2.2	45.24
ID: 1531	AT1G03880	12S seed storage protein CRB	63.52	2.1	
eID: 0005	AT3G21370.1	BGLU19 beta glucosidase 19	63	2.0	59.98
ID: 1073	AT3G21370.1	BGLU19 beta glucosidase 19	48.96	1.9	59.98
ID: 2327	AT5G35590	Proteasome subunit alpha type-6 A	37.4	1.9	27.28
ID: 1465	AT3G02360.2	6-phosphogluconate dehydrogenase family protein	17.9	1.9	53.54
ID: 1220	AT5G44120.3	CRA1, ATCRA1, CRU1 RmlC-like cupins superfamily protein	25	1.9	52.56
eID: 0007	AT5G44120.3	CRA1, ATCRA1, CRU1 RmlC-like cupins superfamily protein	68.01	1.9	52.56
ID: 1602	AT1G03880	12S seed storage protein CRB	63.52	1.8	50.53
ID: 1043	AT3G21370.1	BGLU19 beta glucosidase 19	48.96	1.8	59.98
eID: 0009	AT2G42560.1	late embryogenesis abundant domain-containing protein	50.71	1.7	67.15
ID: 1681	AT5G07440	Glutamate dehydrogenase 2	27.49	1.7	44.67
ID: 1456	AT3G21370.1	BGLU19 beta glucosidase 19	24.29	1.7	59.98

ID: 1064	AT3G21370.1	BGLU19 beta glucosidase 19	48.96	1.6	59.98
ID: 1588	AT5G09810	Actin-7	27.06	1.5	
eID: 0011	AT3G21370.1	BGLU19 beta glucosidase 19	63	1.5	59.98
ID: 1411	AT1G13440.2	GAPC2 glyceraldehyde-3-phosphate dehydrogenase C2	9.35	1.5	41.71
ID: 2542	AT1G03890	12S seed storage protein CRD	8.65	1.5	49.64
eID: 0014	AT1G78900	V-type proton ATPase catalytic subunit A	48.8	-1.5	68.77
ID: 1499	AT3G25860	Dihydrolipoyllysine-residue acetyltransferase component 4 of pyruvate dehydrogenase complex	20	-1.6	50.05
eID: 0032	AT4G09670	Uncharacterized oxidoreductase At4g09670	25.97	-1.6	39.54
ID: 1214	AT4G34200	D-3-phosphoglycerate dehydrogenase 1, chloroplastic	10.45	-1.6	63.29
ID: 1706	AT2G38380	Peroxidase 22	13.75	-1.6	38.08
ID: 2708	AT5G44120	12S seed storage protein CRU1	27.02	-1.7	
ID: 0871	AT5G67360	Subtilisin-like protease SBT1.7	4.76	-1.7	79.37
ID: 0877	AT2G45290	Transketolase-2, chloroplastic	17.27	-1.8	79.87
ID: 0594	AT1G63770	Puromycin-sensitive aminopeptidase	7.13	-1.8	99.10
ID: 0866	Q8RWV0	Transketolase-1, chloroplastic	39.27	-1.8	79.92
ID: 1406	AT5G09590	Heat shock 70 kDa protein 10, mitochondrial	27.42	-2.2	72.95
eID: 0003	ATCG00480	ATP synthase subunit beta, chloroplastic	4.82	-2.3	53.90
ID: 1192	AT2G42560	Late embryogenesis abundant protein	42.36	-2.4	67.15
ID: 1096	AT2G42560	Late embryogenesis abundant protein	13.86	-2.5	67.15
ID: 1622	AT4G05320	Polyubiquitin 10	27.57	-2.6	51.17
ID: 2565	AT5G44120	12S seed storage protein CRU1	27.02	-2.7	
ID: 1185	AT2G42560	Late embryogenesis abundant protein	42.36	-2.9	67.15
ID: 2521	AT1G16030	Heat shock 70 kDa protein	5.57	-3.2	70.87

Supplemental table 2. Primers used to construct GFP fusion construct

Gene	**Primer sequence (5'-3')**
SESA3-F	CGCCCTTGAAACCAAATTAACAT
SESA3-R	AGAAAACATACACAAATAGCAAAAC
GFP-F	GCCATGACGACGCCGTGC
GFP-R	TCAGTGTGACCCATTGAGCCTAG
FGS-F	ATGACGACGCCGTGCCGGAC
FGS-R	CCTCAGTGTGAACCATTGAGCC

Chapter4— NADP−MALIC ENZYME 1 affects germination after seed storage in *Arabidopsis thaliana*

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ABSTRACT

Aging decreases the quality of seeds and results in agricultural and economic losses. The damage that occurs at the biochemical level can alter the seed physiological status. Although loss of viability has been investigated frequently, little information is available on the molecular and biochemical factors that are involved in seed deterioration and loss of seed viability. Oxidative stress has been implicated as a major contributor to seed deterioration and several pathways are involved in protection against this. In this study, we show that seeds of *Arabidopsis thaliana* lacking a functional *NADP-MALIC ENZYME 1* (*NADP-ME1*) gene have reduced seed viability relative to wild type. Seeds of the *nadp-me1* loss-of-function mutant display higher levels of protein carbonylation than wild type. NADP-ME1 catalyses the oxidative decarboxylation of malate to pyruvate with the simultaneous production of CO_2 and NADPH. Upon seed imbibition malate and amino acids accumulate in embryos of aged seeds of the *nadp-me1* loss-of-function mutant compared to wild type. *NADP-ME1* expression is increased in imbibed aged- as compared to non-aged seeds. NADP-ME1 activity at testa rupture promotes normal germination of aged seeds. In seedlings of aged seeds NADP-ME1 is specifically active in the root meristematic zone. We propose that through the production of NADPH, NADP-ME1 is required for protecting seeds against oxidation during seed dry storage.

To be submitted

INTRODUCTION

Seed survival in the period between seed dispersal and germination is an adaptive strategy that plants use to maintain their life cycle. Longevity mechanisms allow the seed to survive prolonged periods in the (often dry) soil seed bank. Aging, occurring during inappropriate conditions or prolonged dry storage, dramatically affects germination and vigour. Genetic, genomic, and postgenomic analyses using the model plant *Arabidopsis thaliana* have revealed a major role of reactive oxygen species (ROS) or oxidative stress in the process of seed aging. At low to moderate concentrations, ROS serve as signalling molecules, whereas at high concentrations they cause damage to biomolecules. This includes peroxidation of lipids, oxidation of proteins, damage to nucleic acids, enzyme inhibition, activation of programmed cell death pathways, and, ultimately, cell death (Bailly et al., 2008). Protein oxidation, especially carbonylation, induces loss of functional properties of proteins and/or enhances their susceptibility towards proteolysis (Davies, 2005; Rajjou et al., 2008). In order to optimize seed life span plants evolved complex systems of protection, detoxification and repair. Scavenging or detoxification of excess ROS is achieved by an efficient antioxidative system that is comprised of non-enzymatic, as well as enzymatic antioxidants (Noctor & Foyer, 1998).

Accordingly, mutations in genes encoding proteins, which are involved in ROS accumulation or protection against ROS production, affect seed longevity. *FERRIC-CHELATE REDUCTASE 1*, a mutant in mitochondrial NADH dehydrogenase shows constitutive ROS accumulation and its seeds are very sensitive to aging (Clerkx et al., 2004). Vitamin E (tocopherols and tocotrienols) act as antioxidant and prevent non-enzymatic lipid oxidation. Mutations in genes involved in tocopherol biosynthesis (*vte1* and *vte2*) also result in a decrease of seed longevity (Sattler et al., 2004). The reduced longevity of *transparent testa* mutants (Debeaujon et al., 2000), which are altered in the flavonoid synthesis pathway, is in agreement with the protective role attributed to the testa. Repair of damaged proteins by methionine sulfoxide reductases from *Medicago truncatula* and PROTEIN L-ISOASPARTYL METHYLTRANSFERASE from Arabidopsis, rice and lotus (*Nelumbo nucifera*) could enhance seed vigor and longevity (Chatelain et al., 2013; Oge et al., 2008; Petla et al., 2016; Verma et al., 2013). Moreover, suppression of

phospholipase D in Arabidopsis decreases the loss of unsaturated fatty acids and oxidative stress and enhances seed quality and viability (Devaiah et al, 2007). Seed longevity is induced during seed maturation (Chatelain et al., 2012; Probert et al., 2009; Righetti et al., 2015). Therefore, mutants affected in seed development, such as *LEAFY COTYLEDON 1, LEAFY COTYLEDON 2, FUSCA 3* (Meinke et al., 1994) and *ABSCISIC ACID INSENSITIVE 3* (*ABI3*) but also *DELAY OF GERMINATION* (Bentsink et al., 2006; Dekkers et al., 2016) have poor seed longevity.

Recently, a quantitative genetic analysis has revealed 12 loci involved in seed longevity after natural aging (Nguyen et al., 2012). Proteome analysis performed on after-ripened and 4-year-old (aged) dry seeds, from Arabidopsis introgression lines containing these different seed longevity loci, identified several proteins involved in seed longevity after accelerated aging (Nguyen et al., 2015). Among these, NADP-MALIC ENZYME 1 (NADP-ME1) displayed lower abundance in aged seeds than in after-ripened seeds of the near isogenic line with the lowest longevity (Nguyen et al., 2015). Moreover, a *NADP-ME1* knockout mutant showed reduced seed longevity when compared to wild type (Yazdanpanah et al., 2017).

NADP-ME (EC 1.1.1.40) catalyses the oxidative decarboxylation of malate, producing pyruvate, CO_2, and NADPH. In Arabidopsis, four genes encode NADP-ME (*NADP-ME1*, At2g19900; *NADP-ME2*, At5g11670; *NADP-ME3*, At5g25880; and *NADP-ME4*, At1g79750) (Gerrard Wheeler et al., 2005; Maurino et al., 2009). NADP-ME4 is localized to plastids, while NADP-ME1 to -3 are localized to the cytosol (Gerrard Wheeler et al., 2009; Gerrard Wheeler et al., 2005). Arabidopsis NADP-ME isoforms show amino acid identities between 78% and 90% but they differ in their expression patterns and biochemical properties (Gerrard Wheeler et al., 2009; Gerrard Wheeler et al., 2008; Gerrard Wheeler et al., 2005; Maurino et al., 2009). NADP-ME4 is constitutively expressed in vegetative and reproductive organs. The expression of NADP-ME3 is high and restricted to trichomes, stipules, and pollen grains in the late maturation stages. NADP-ME2 is constitutively expressed in vegetative organs and is responsible for the bulk of NADP-ME activity in leaves. This isoform plays a role during the basal defence response, where it is required for the production of ROS following pathogen recognition (Voll et al., 2012). NADP-ME1 shows the lowest catalytic efficiency for both malate and NADP and is highly

expressed in radicle tips during germination, in some secondary roots in adult plants and in the embryo at the latest stages of embryogenesis (Gerrard Wheeler et al., 2005).

Here, using single and multiple NADP-ME loss-of-function mutants and a combination of biochemical and physiological methods we investigated the participation of NADP-ME1 in seed germination. We show that seeds lacking a functional NADP-ME1 have reduced seed longevity and more severe protein carbonylation than wild type. We propose a role for NADP-ME1 in the protection of seed proteins against oxidative damage through the production of NADPH.

MATERIALS AND METHODS

Plant material

Homozygous T-DNA insertion mutants *nadp-me1* (SALK-036898), *nadp-me2-1* (SALK-073818), *nadp-me2-2* (SALK-020607), *nadp-me3* (SALK-139336), *nadp-me4-1* (SALK-064163), and *nadp-me4-2* (Gabi-KAT line GK371F05) were isolated previously (Gerrard Wheeler et al., 2005) by a PCR-based reverse genetic screen for T-DNA insertions in the corresponding genes. The mutant lines are in the Columbia-0 (Col-0) background. Homozygous double and triple NADP-ME loss-of-function mutants were obtained by genetic crossing of the homozygous lines *nadp-me1*, *nadp-me2-1*, *nadp-me-3*, and *nadp-me4-1* (Gerrard Wheeler et al., 2005).

Constructs and plant transformation

Complementation cloning and overexpression analyses have been performed with *NADP-ME1* under a seed specific promoter (SESA3; At4g27160). All construct parts have been amplified by PCR using Phusion Flash High-Fidelity PCR Master Mix (Life Technologies). The final construct was built by overlapping PCR, digestion and ligation (primers can be found in Supplemental Table S1). The SESA3 promoter was cloned into pAlligator2. The *NADP-ME1* coding region was amplified on cDNA of Arabidopsis. The terminator amplicon was amplified on gDNA of Arabidopsis. The terminator amplicon was used in overlapping PCR in combination with the *NADP-ME1* amplicon. This product was the template for a second overlapping PCR that combined the coding region and the terminator with the promoter. The full-length construct was restricted using the *NotI* restriction enzyme (Fermentas Fast Digest),

cleaned with Zymogen DNA Clean & Concentrator (Baseclear), and ligated into pGII 0227-R4R3. *Escherichia coli* DH5α cells were transformed using a standard heat-shock protocol and transformants were selected on LB-kanamycin agar (50 μg.ml^{-1}). Plasmids were extracted from the transformants and verified by PCR. A plasmid bearing the construct was transformed into Col-0 and *nadp-me1* plants with *Agrobacterium tumefaciens* (GV3101; Koncz & Schell, 1986) using the floral dip method (Clough & Bent, 1998). Transgenic lines were selected on 30 μg ml^{-1} hygromycine plates.

Germination Assays

Germination experiments were performed as described by Joosen *et al*. (2010) with small adjustments. In brief, two layers of blue germination paper were equilibrated with 48 ml demineralized water in plastic trays (15 × 21 cm). 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 twice a day for a period of 7 days using a Nikon D80 camera fixed to a repro stand with a 60 mm macro objective. The camera was connected to a computer with Nikon Camera Control Pro software version 2.0. Percentages germinated seeds and rate of germination (T50) were calculated.

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 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. Stress conditions were: osmotic stress (-1 MPa mannitol; Sigma-Aldrich), salt stress (130 mM NaCl; Sigma-Aldrich), ABA stress (0.15 μM 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 ZnSO4 solution (40°C, 85% relative humidity) in a closed tank for 2 or 5 days (ISTA, 2012). The most discriminative incubation

time was presented. After incubation the seeds were taken out and germinated on demineralized water as described before.

***NADPH-ME1* expression**

RNA was isolated using the Nucleospin RNA plant kit (Macherey-Nagel: 740949) according to the manufacturer's protocol with minor modifications: 3-5 mg freshly harvested seeds were used for the extraction. Lysis was performed using 315 µL buffer RAP + 35µL Plant RNA Isolation Aid (Ambion: AM9690) + 3.5µL β-Mercaptoethanol (Sigma: M6250). Final RNA was eluted in 40µL RNAse free water. Quality and concentrations were measured by loading 2µL 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 750ng RNA using the iScript cDNA Synthesis Kit (Bio-Rad: 170-8890) according to the manufacturer's protocol. cDNA was diluted 10 times with sterile milliQ water. For each sample 2.5µL cDNA, 5µL iQ SYBR green supermix (Bio-Rad: 172-5125) and 0.5µL primer mix (10µL work solution) were added and supplemented with water to 10µL. RT-qPCR was performed on a CFX connect (Bio-Rad).

Primer pairs used were as follows: NADP-ME1 forw: 5´ TCGGTAGAGGAAAGCCGCAA -3´ and NADP-ME1 rev: 5´ACTTGTCCAGCTAATGCCTCAG-3´. NADPH-ME1 expression was normalized by the expression of two reference genes that are stably expressed in dry seeds: At4g12590 and At4g34270 (Dekkers et al., 2012). Expression was calculated by using qbasePLUS (Hellemans et al., 2007) which is commercially available software (Biogazelle, Ghent, Belgium, www.biogazelle.com).

***In situ* NADP-ME activity assay**

Embryos isolated from whole seeds, non-aged (100% germination) and aged (accelerated aged, 40-80% germination), at different time-points during germination (1HAS, 6HAS, testa rupture (TR) endosperm rupture (ER) and embryos at the root hair stage) and full grown seedlings were fixed in 2% paraformaldehyde with 2% polyvinylpyrrolidone 40 and 0.001 M DTT, pH 7.0, at 4°C for 1 h. After fixation, seedlings were rinsed overnight in water at 4°C and

refreshed at least five times to remove soluble carbohydrates (Sergeeva *et al*., 2004). Staining for NADP-ME activity was performed according to (Baud & Graham, 2006) with modifications. Plant material was submerged in an incubation mixture containing 50 mM HEPES-KOH (pH 7.4), 1 mM NADP, 5 mM L-malate, 10 mM MgSO4, 0.4 mM PMS and 0.8 mM nitroblue tetrazolium (NBT), for 60 min at 30°C. The enzymatic reaction was stopped by rinsing embryos and seedlings in distilled water. Plant material could be stored at 4°C in water for at least 1 month without loss of staining. Photos were taken by Nikon 80i and Stereo 60x.

Metabolite measurements using GC-MS

Lyophilized seed material was extracted for metabolite analysis by chloroform:methanol:water (1:2.5:1) and analysed by gas chromatography-mass spectrometry (GC-MS) according to Fiehn and Kind (2006) using a 7890B GC coupled to a 7200 QTOF (Agilent, USA). Data analysis was conducted with the Mass Hunter Software (Agilent, USA). For relative quantification, all metabolite peak areas were normalized to the peak area of the internal standard ribitol (Sigma-Aldrich, USA) added prior to extraction. Statistical analysis was done using Microsoft Excel, and R statistics (version 3.2.3.; provided by the CRAN projecthttp://www.Rproject.org). For the two-way ANOVA followed by a Tukey HSD post-hoc test, we used the following functions: anova(), lm(), TukeyHSD(), aov().

Detection of protein carbonylation

Carbonylated protein profiles were determined by 1D PAGE of total protein extract followed by derivatization with 2,4-dinitro-phenylhydrazine and immunological detection of the DNP adducts with monoclonal anti-DNP antibody (OxyBlot Oxidized Protein Detection Kit; Chemicon) as described previously (Job et al., 2005).

Quantification of protein carbonylation

Semi-quantitative data of protein carbonyls were obtained from Western blot analysis with ImageQuant software. These values are expressed in arbitrary units and are a result of protein stain density per anti-DNPH-signal density. The measured values were normalized to average values. Values are means ±SD of three replicates.

RESULTS

Lack of a functional NADP-ME1 results in reduced seed longevity

To study the effect of NADP-ME1 in seed germination, knock-out mutants (*nadp-me1*) have been analysed for several germination parameters. Compared to wild type, the *nadp-me1* mutant displays severely reduced seed longevity, higher sensitivity to germination in the osmoticum mannitol, and reduced dormancy (Fig. 1). The reduced germination of aged seeds is mainly reflected by the lower germination capacity (Fig. 1C) and not by significantly reduced germination rates (Supplemental Fig. S1). Germination occurs in two phases; first the testa splits (testa rupture; TR) and, thereafter, the radicle (embryonic root) protrudes the endosperm (endosperm rupture; ER), completing germination *sensu stricto*. The reduced germination capacity of *nadp-me1* after aging is reflected both by reduced testa rupture as well as endosperm rupture (Supplemental Fig. S2). To analyse whether the reduced longevity is specific for the lack of NADP-ME1, loss-of-function mutants of the other three Arabidopsis NADP-ME isoforms, as well as double and triple combinations of the mutant lines, were also analysed. This analysis showed that only *nadp-me1* and double and triple mutant combinations with *nadp-me1* had reduced seed longevity compared to wild type (Fig. 1C). Moreover, complementation of the *nadp-me1* mutant with the wild type *NADP-ME1* under the seed specific promoter (SESA3; At4g27160) reverted the mutant phenotype (Fig. 1D). Furthermore, the overexpression of *NADP-ME1* in the wild type background lead to increased seed longevity (Fig. 1D). Therefore, the seed longevity effects are specific to NADP-ME1 functioning.

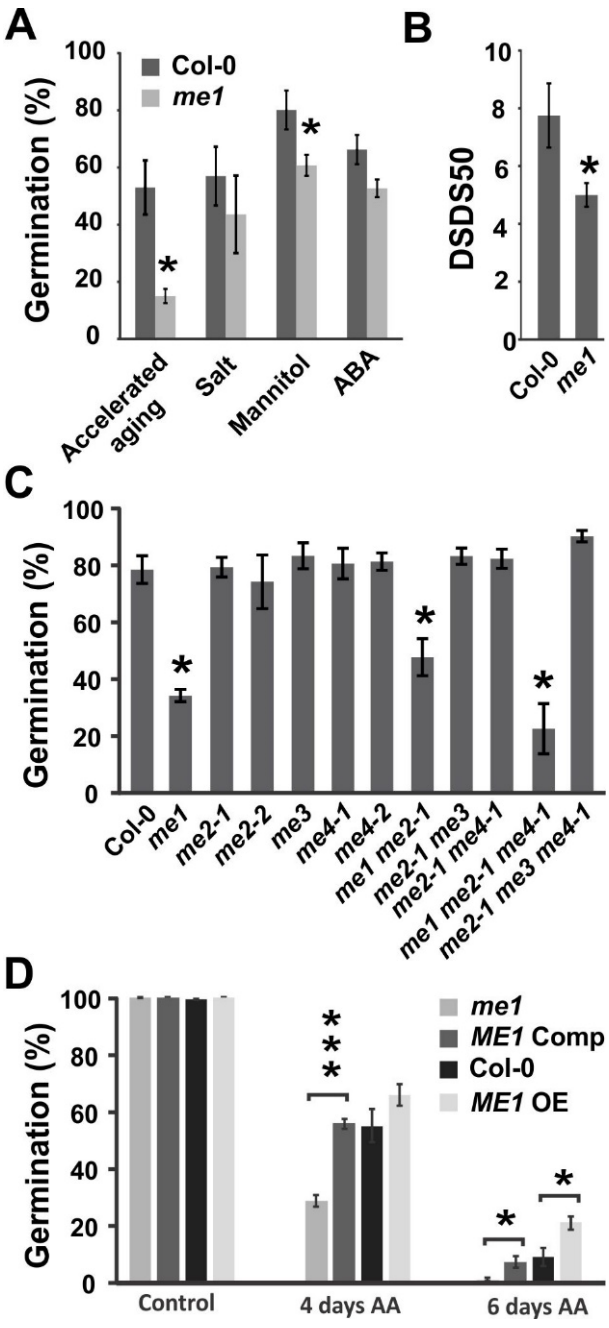

Figure 1. Role of NADP-ME1 in seed performance. A) Germination of after ripened *nadp-me (me) 1* and Columbia-0 (Col-0) seeds after accelerated aging, in salt (130mM), mannitol (-1MPa) and ABA (0.15 μM). These seeds germinated to 100% in water. B) Seed dormancy levels expressed as days of seed dry storage to reach 50% of germination (DSDS50) of *me1* and Col-0. C) Germination after 6 days of accelerated aging of *me1*, *me2-1, me2-2*, *me3-1*, *me4-1*, *me4-2* their double (*me1 me2-1*; *me2-1 me3* and *me2-1 me4-1*) and triple (*me1 me2-1 me4-1* and *me2-1 me3 me4-1*) mutants. D) Germination after 0 (Control), 4 and 6 days of accelerated aging (AA) of *me1*, the *me1* complementation line (*ME1* Comp) containing SESA3:NADP-ME1, Col-0 and the *ME1* overexpressor line (*ME1* OE) containing SESA3:NADP-ME1 in the Col-0 genetic background. Shown are averages of four biological replicates and their SE. Significant differences with wild type Col-0 determined by Tukey HD test are indicated (* $P < 0.05$), *** $P < 0.001$).

***NADP-ME1* expression is increased in aged seed**

NADP-ME1 expression analyses over plant development revealed that it is expressed in late stages of embryogenesis, in the radicle during germination and in seedling roots (Gerrard Wheeler et al., 2005). Here, we performed a detailed analysis of *NADP-ME1* expression in seeds by qRT-PCR. *NADP-ME1* is first detected at eight days after pollination and peaks during late seed maturation (Fig. 2A). *NADP-ME1* transcript levels remained detectable in freshly harvested dry seeds, but were reduced during seed storage (after-ripened seeds; dry). Transcript abundance was also reduced upon seed imbibition, but was still detectable at 12 hours after sowing (HAS) (Fig. 2).

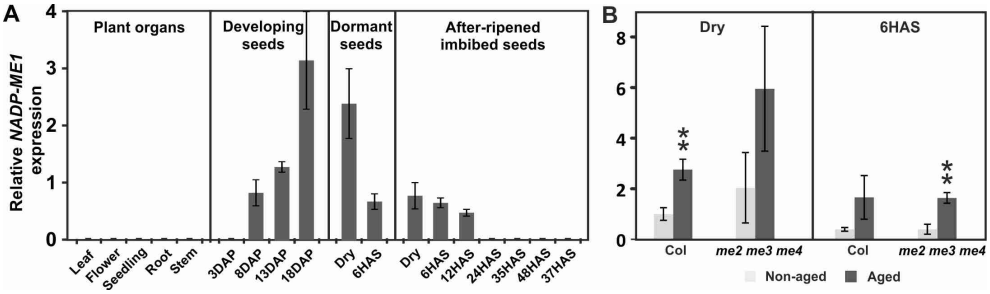

Figure 2. Relative expression of *NADP-ME1*. A) RT-qPCR results for *NADP-ME1* during plant development in the Col-0 wild type; leaves, flowers, seedlings, roots, stems, siliques at 3, 8, 13 and 18 days after pollination (DAP), in dormant dry seeds (Dry) and 6 hours after sowing (6HAS), along the germination time course in after-ripened dry and 6, 12, 24, 35, 48 and 73 HAS. B) RT-qPCR results in non-aged and aged seeds (accelerated aging) in four year old seeds, dry and 6 HAS, of Col-0 and the *nadp-me2-1 nadp-me3 nadp-me4-1* (*me2 me3 me4*) triple mutant. It should be noted that the aged dry and 6 HAS samples consist of a mixture of seeds that are able to germinate and those that are not. Shown are averages of 3-4 biological replicates and their SE. Significant differences between non-aged and aged determined by t-test are indicated (** $P < 0.01$).

The effect of accelerated aging on relative *NADP-ME1* levels was analysed in embryos of non-aged and aged seeds of Col-0 and the *nadp-me2-1 nadp-me3 nadp-me4-1* triple mutant. For both time points (dry seeds and at 6 HAS) and both genotypes we identified relatively more *NADP-ME1* transcripts in aged seeds than in non-aged seeds, even though the aged seeds contained a considerable number of non-germinating (dead) seeds (Fig. 2B, Supplemental Fig. S2 [Col-0 47%, triple mutant 20% dead seeds]). This increase of relative *NADP-ME1* transcript levels can

be explained by either transcriptional activity during the accelerated aging treatment or by the relative higher stability of *NADP-ME1* transcripts compared to that of the reference genes. The fact that mRNA levels dropped during seed dry storage (comparing dormant dry with after-ripened dry seeds in Fig. 2A) suggests the former. Moreover, we added the *nadp-me2-1 nadp-me3 nadp-me4-1* triple mutant to these analyses as they better withstand the artificial aging treatment (Supplemental Fig. S2). The better germination of the triple mutant cannot be explained by a higher *NADP-ME1* expression, as we found similar expression levels in Col-0 and the triple mutant (Fig. 2).

NADP-ME1 enzyme activity in germinating non-aged and aged seeds

To investigate whether there is NADP-ME1 activity during seed germination, *in situ* NADP-ME activity assays were performed on germinating non-aged and aged seeds of Col-0, the *nadp-me2-1 nadp-me3 nadp-me4-1* triple mutant and *nadp-me1* (Fig. 3). NADP-ME activity during germination (from 1 HAS until fully developed seedlings) is largely accounted for by NADP-ME1, as indicated by the reduced NADP-ME activity in *nadp-me1* seeds. NADP-ME1 activity during seed imbibition is highly dynamic (Fig. 3A-C). It is high at 1HAS, which is probably a left-over from the high activity during seed maturation (Gerrard Wheeler et al., 2005) and is largely reduced at 6 HAS. At testa rupture (TR) NADP-ME1 activity is high and it is reduced again at endosperm rupture (ER). NADP-ME activity is much higher in aged seeds. Quantification of the NADP-ME activity revealed that it is as high in the *nadp-me2-1 nadp-me3 nadp-me4-1* triple mutant as in Col-0. In the triple mutant however, the activity is only accounted for by NADP-ME1, which probably results in the better germination of the *nadp-me2-1 nadp-me3 nadp-me4-1* triple mutant seeds. The variation observed per sample point is expected from the inherent intrinsic variation that exists among individuals within a seed lot, i.e. the varying age of seeds harvested from one plant and differences in rate of aging during the artificial aging treatment.

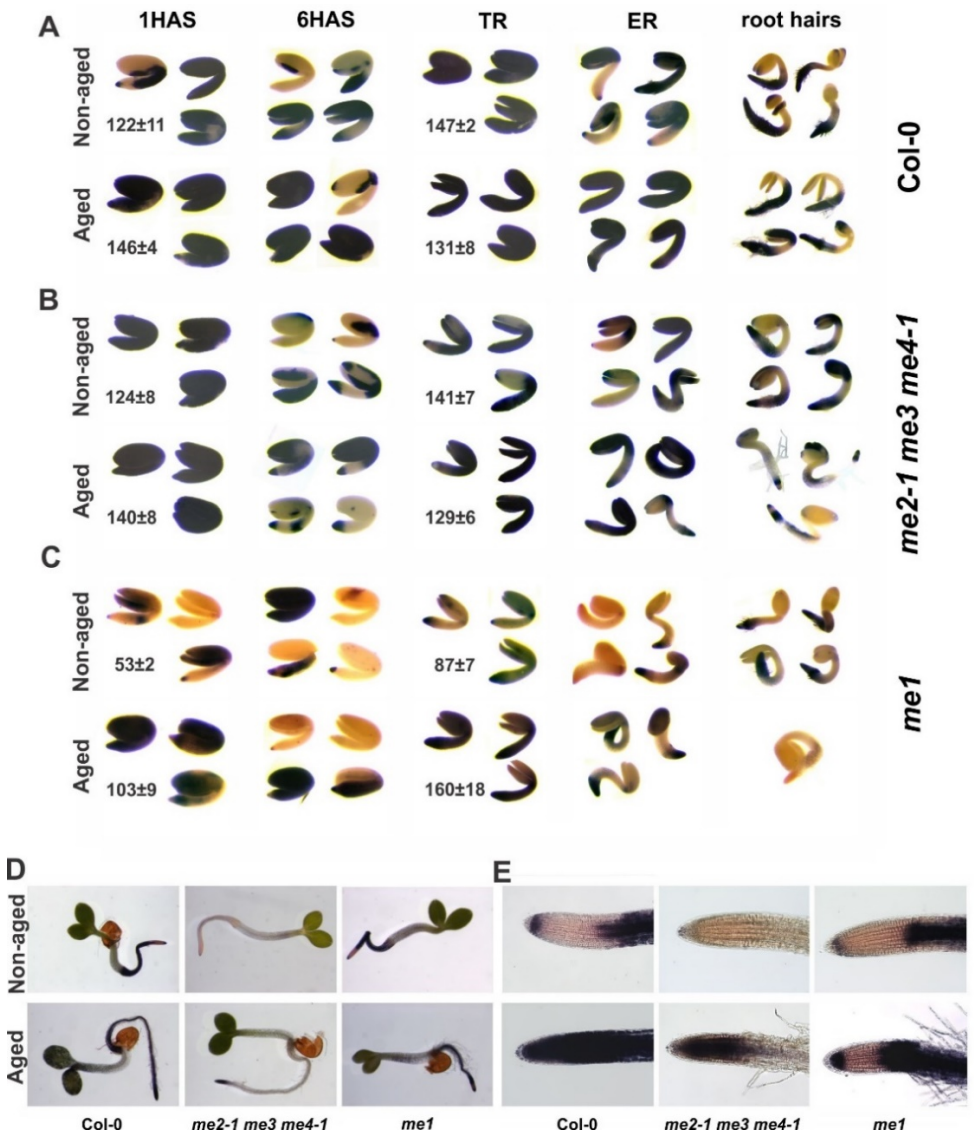

Figure 3. In situ NADP-ME activity assay. Activity staining was performed in non-aged and aged (accelerated aging) embryos and seedlings of Col-0, the *nadp-me (me) 2-1 me3 me4-1* triple mutant and *me1*. A-C) Up to four embryos per imbibition state (1HAS, 6HAS, testa rupture (TR), endosperm rupture (ER) and the stage at which the root hairs appear) are shown. At 1HAS and TR NADP-ME activity has been quantified. Average relative pixel intensities at the radicle tip of 10 embryos and their SE are presented. It should be noted that the aged 1 and 6 HAS embryos consist of both embryos that are able to germinate and those that are not. Evidentially the embryos at TR and ER did germinate. A) Col-0; B) *me2-1 me3 me4-1*; C) *me1*; D) Activity in whole seedling; E) Close up of the root tip. A larger magnification of the root tip can be found in Supplemental Fig S3.

In seedlings of aged seeds NADP-ME1 is active in the root meristematic zone. Col-0 and *nadp-me2-1 nadp-me3 nadp-me4-1* triple mutant seedlings showed NADP-ME activity in this region, but activity was absent in *nadp-me1* seedlings (Fig. 3DE, Supplemental Fig. S3). NADP-ME1 activity was almost absent in the root meristematic zone of non-aged seeds, as seeds of the *nadp-me2-1 nadp-me3 nadp-me4-1* triple mutant and Col-0 lack activity in this region (Fig. 3DE). These results indicate that NADP-ME1 is active in the root meristematic zone of seedlings from aged seeds.

Metabolic changes in non-aged and aged seeds of NADP-ME loss-of-function mutants

NADP-ME catalyses the oxidative decarboxylation of malate to pyruvate, producing CO_2 and NADPH. To gain a better understanding of the influence of NADP-ME1 on the metabolic status during seed germination we compared the relative amounts of malate and other metabolites in seeds of the single loss-of-function mutants of the four Arabidopsis NADP-ME isoforms and the wild type at different stages during germination (dry seed, 6 HAS, radical protrusion and seedling) either non-aged or aged. The relative amount of malate in non-aged *nadp-me1* seeds did not differ from the wild type or the other NADP-ME loss-of-function mutants. However, malate was elevated in aged *nadp-me1* seeds at 6 HAS and at radical protrusion stages (Fig. 4). This result is in agreement with the fact that the activity of NADP-ME1 increased during germination of aged wild type seeds (Fig. 3A).

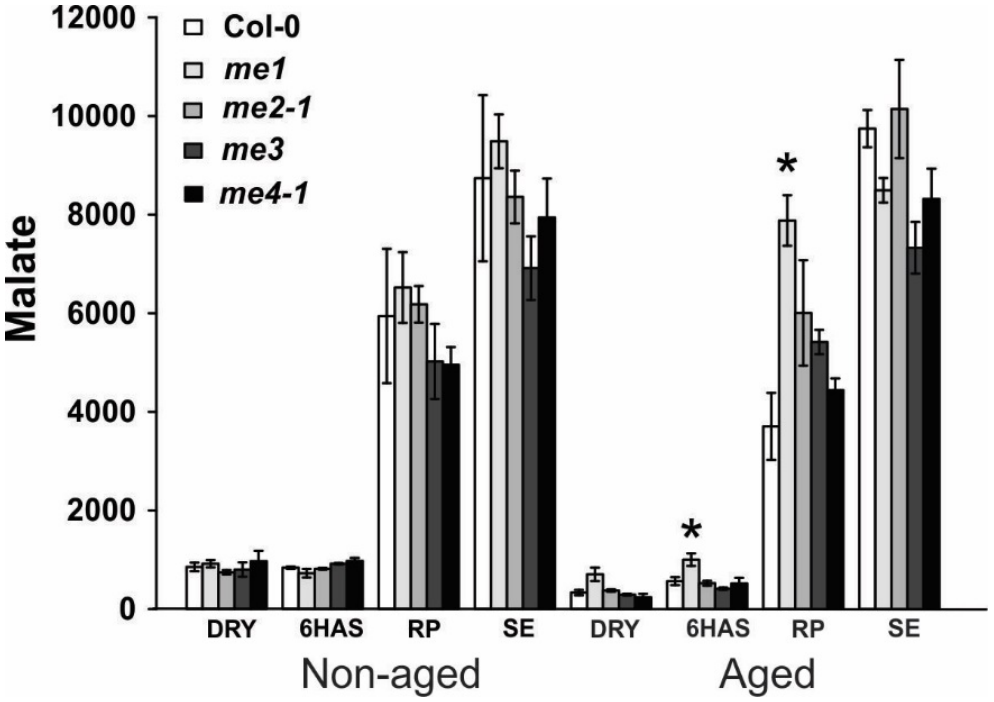

Figure 4. Malate levels in non-aged and aged seeds. Relative malate levels in non-aged and aged (by accelerated aging test) seeds at four different stages during germination; dry seeds (DRY), 6 hours after sowing (6HAS), seeds at radical protrusion (RP) and seedlings (SE) of wild type (Col-0) and the *nadp-me* (*me*) single mutants (*me1*, *me2-1*, *me3* and *me4-1*). The aged batches of dry seeds and seeds at 6 HAS also contained seeds that do not germinate. Amino acid levels were measured by GC-MS (see Material and Methods for details). Values are presented as mean ± SE (n = 3 - 4). An ANOVA with a post-hoc analysis by Tukey's HSD was applied. Asterisks indicate significant differences relative to the respective wild type ($p < 0.05$). For differences between non-aged and aged seeds see Table S1.

Seeds of *nadp-me1* at 6 HAS accumulated amino acids at much higher levels than the wild type and the other *NADP-ME* loss-of-function mutants (Fig. 5 and Supplemental Table S1). Among these amino acids, alpha-alanine, cysteine, glutamic acid, glycine, isoleucine, leucine, methionine, ornithine, phenylalanine, serine, threonine and valine showed relative amounts that were more than three-fold higher than the wild type. The enhanced accumulation of these free amino acids suggests either enhanced protein degradation in aged *nadp-me1* seeds at 6 HAS or a lower translational capacity leading to a reduced incorporation of free amino acids into newly synthesized proteins.

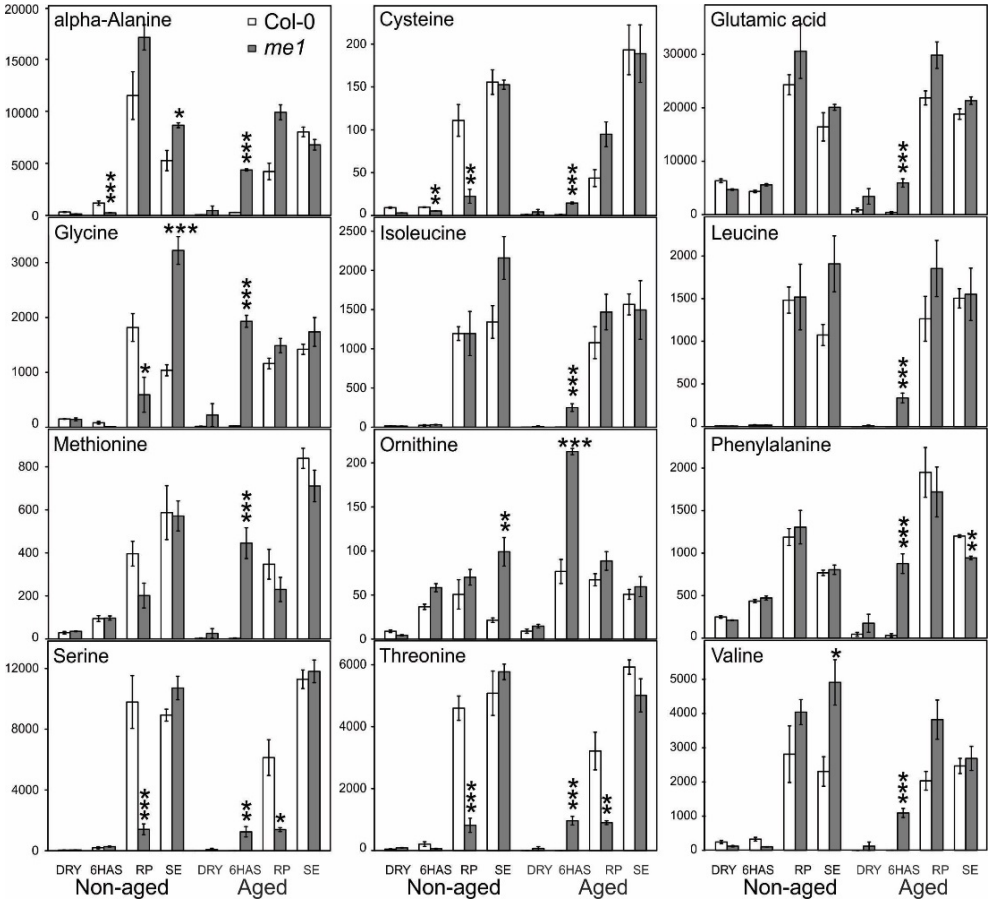

Figure 5. Higher amino acid abundance in aged *nadp-me1* seeds. Relative metabolite amounts * mg DW^{-1} in wild type (Col-0), *me1*, *me2-1*, *me3* and *me4-1* at four different stages (DRY, 6HAS, RP, SE) during seed germination of non-aged seeds and seed aged by accelerated aging test. The aged batches of dry seeds and seeds at 6 HAS also contained seeds that do not germinate. Amino acid levels were measured by GC-MS (see Material and Methods for details). Values are presented as mean ± SE (n = 3 - 4). An ANOVA with a post-hoc analysis by Tukey's HSD was applied. Asterisks indicate significant differences relative to the respective wild type (three asterisks, $p < 0.001$; two asterisks, $p < 0.01$; one asterisk, $p < 0.05$). For differences between non-aged and aged seeds see Table S1.

A more detailed look at the other primary metabolites learns that also these were mostly affected in aged *nadp-me1* seeds at 6 HAS (Supplemental Table S1, http://www.wageningenseedlab.nl/thesis/fyazdanpanah/SI/). It is worth mentioning that when working with seeds of different age, the aged batches will always contain seeds that would not germinate. For the samples at radical protrusion and the seedling stage, the non-germinating seeds have been excluded from the analyses. For dry seeds and seeds at 6 HAS this is not possible as we cannot predict whether a seed will germinate or not.

The absence of NADP-ME1 activity enhances the oxidation of seed proteins leading to reduced germination

We hypothesized that NADP-ME1 is involved in redox homeostasis through the production of NADPH during germination. A non-functional NADP-ME1 would thus lead to redox misbalances, enhanced oxidative stress and reduced viability. To determine whether aged seeds of plants lacking a functional NADP-ME1 display increased oxidation, the carbonylation profile of the seed proteome was investigated in dormant-, after ripened- and aged seeds of *nadp-me1* and wild type. Western-blotting after 1D gel electrophoresis allows revealing the most abundant carbonylated proteins, which are indicative of the oxidation state of the whole proteome. The total protein profile was similar in both *nadp-me1* and wild-type seeds (Fig. 6A). However, the proteins, largely seed storage proteins, were more carbonylated in *nadp-me1* seeds than those of the wild type (Fig 6B and C). This phenomenon was already visible in freshly harvested dormant seeds and became more pronounced upon further storage (Fig. 6B and C).

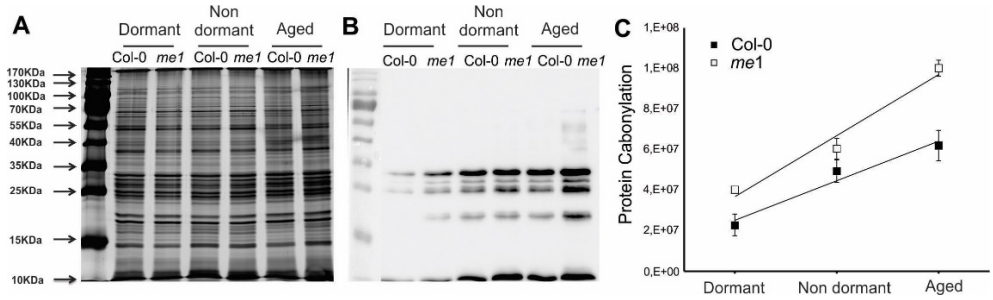

Figure 6. Protein carbonylation in seeds with different storage times. Protein carbonylation of seed proteins in dormant, non-dormant (after-ripened) and accelerated aged (Aged) seeds. A) 1D gel electrophoresis stained with Coomassie Brilliant Blue of total seed protein extracts from Col and the *nadp-me1* (*me1*) mutant. B) Carbonylated proteins as detected by immunodetection of protein-bound DNP after derivatization with hydrazine. C) and semi-quantitative data of protein carbonylation. The Y axis corresponds to the relative abundance of carbonylated protein, which is an arbitrary unit. It has been calculated using the signal of the western-blot (Fig 6B) and is normalized by the total protein signal (Fig 6A) in each lane using ImageQuant software.

Discussion

Seed longevity is based on the protection of the embryo during storage but also on the way the embryo can cope with damage when germinating. Here, we show that NADP-ME1 is important for the germination of aged seeds. Only Arabidopsis mutant seeds deficient in NADP-ME1, but not the ones lacking one or more of the other three NADP-ME isoforms (NADP-ME2, -3 or -4), showed reduced longevity. Previous studies have shown that NADP-ME1 is highly expressed in the embryo during seed maturation and late embryogenesis (Gerrard Wheeler et al., 2005) and that the kinetics and structural properties of NADP-ME1 are very different from the properties of other plant NADP-ME isoforms (Gerrard Wheeler et al., 2005; Maurino et al., 2009). In this work, we measured aging of seeds as the germination capacity after accelerated aging (ISTA, 2012). Studies on seed longevity depend on artificial methods due to the relatively long storability of seeds. After four years of dry storage Col-0 and the *nadp-me2 napd-me3 nadp-me4* triple mutant still germinated at more than 90% (Supplemental Fig. S2), however, the rate of germination was reduced compared to fully after-ripened seeds (Supplemental Fig. 1). Moreover, accelerated aging for only two days was enough to severely reduce germination of the four-year-old seeds, while fresh seeds required six days for the

same reduction in germination. Thus, seed dry storage increased the sensitivity to the aging test. The results presented here revealed differences between aging due to seed dry storage and to accelerating aging, i.e. relative *NADP-ME1* mRNA levels (Fig. 2). Likely, oxidative stress in accelerating aging conditions is more compared to dry conditions and seeds might even transcribe mRNAs in accelerating aging conditions considering the relative increase in *NADP-ME1* transcripts. Nevertheless, accelerated aging resulted in lower germination levels of *nadp-me1* mutant seeds compared to wild type and this coincides with the increased NADP-ME1 activity in germinating seeds.

We show that NADP-ME1 activity is localized to the root meristematic region in establishing seedlings (Fig. 3DE, Supplemental Fig. S3). Both, cell proliferation and cell elongation, are important to maximize growth after germination (Beemster & Baskin, 1998; Ubeda-Tomas et al., 2009). Likely, NADP-ME1 activity in the meristematic region is important for the establishment of the seedling.

NADP-MEs catalyse the oxidative decarboxylation of malate to pyruvate and CO_2. The higher malate levels in some developmental stages of aged *nadp-me1* seeds compared to wild type would be a consequence of the absence of NADP-ME1 activity in this mutant. Moreover, the high NADP-ME1 activity in *nadp-me2-1 nadp-me3 nadp-me4-1* triple mutant seeds, could be caused by higher NADP-ME1 protein levels. It is unknown if the higher activity of NADP-ME1 in imbibed aged seeds is required for the metabolic conversion of malate or whether it serves another role, e.g. to produce reducing power in the form of NADPH. NADP-ME1 produces pyruvate in the cytosol, which would be further metabolized to acetyl-CoA to feed the glyoxylate cycle or to phosphoenolpyruvate and oxaloacetate to feed the TCA cycle (Maurino & Engqvist, 2015). At the same time, NADPH produced in the reaction could be involved in optimizing the seed redox balance. It is known that a main function of Arabidopsis NADP-ME2 is to contribute to the provision of cytosolic NADPH to meet the demands of reducing power during basal defence responses (Voll et al., 2012). Also in other kingdoms cytosolic NADP-ME is required for the provision of NADPH, e.g. the human NADP-ME isoforms play an important role in lipogenesis by providing NADPH for the biosynthesis of long-chain fatty acids and steroids (Loeber et al., 1994). To investigate whether NADP-ME1 could play a role

in seed redox balance we investigated the levels of protein carbonylation during dry storage of wild type Col-0 and *nadp-me1* seeds. These analyses clearly showed that carbonylation of seed proteins is enhanced in *nadp-me1*, indicating that NADP-ME1 is required for protecting seeds against oxidation during seed dry storage (aging). Considering the expression of NADP-ME1 during seed maturation the foundation of this protection might already have been laid during this developmental stage. This is also in agreement with the high NADP-ME1 activity measured at 1 HAS and its reduction upon longer imbibition (6 HAS). Unfortunately, we were not able to identify differences in ROS levels between non-aged and aged germinating *nadp-me1* seeds (data not shown). This might be explained by the fact that we could only analyse germinating seedlings that survived the aging treatment, most probably through the production of reducing power by other means.

Protein carbonylation is the most prevalent type of protein oxidation caused by ROS. Studies in sunflower (*Helianthus annuus*) and Arabidopsis seeds suggest that the oxidation of specific proteins play a role in the release of seed dormancy (Job et al., 2005; Oracz et al., 2009; Oracz et al., 2007). It is hypothesized that for successful germination an optimal level of ROS is required (Bailly et al., 2008). Too little oxidation suppresses seed germination (e.g. in dormant seeds), and too much leads to cellular oxidative damage and eventually seed deterioration. This role for oxidation fits with both the reduced dormancy (quicker after-ripening) and reduced longevity of the *nadp-me1* mutant seeds. Likely, higher levels of ROS in dormant seeds of *nadp-me1* in comparison to the wild type result in reduced dormancy of the mutant. On the contrary, aged *nadp-me1* seeds cannot tolerate ROS over-accumulation; and the higher protein carbonylation in these seeds is translated in the failure to germinate.

Since carbonylated proteins are destined to degradation (El-Maarouf-Bouteau et al., 2013), this can explain, at least in part, the relative increase of the amount of most free amino acids in *nadp-me1* at 6 HAS. A similar phenomenon was observed in seeds of plants lacking cytosolic ASCORBATE PEROXIDASE 6, a hydrogen peroxide-scavenging enzyme, in which the levels of most amino acids and some organic acids were also significantly enhanced (Chen et al., 2014). Moreover, seeds

of this mutant accumulate higher levels of ROS, exhibit increased oxidative damage, and display reduced germination on soil. Nevertheless, we cannot rule out an effect of the metabolic misbalance in *nadp-me1* seeds on the accumulation of amino acids.

In the Arabidopsis seed proteome three protein forms of NADP-ME1 were identified (Galland et al., 2012). The major NADP-ME1 form was highly carbonylated in aged dry seeds that have lost their germination ability (Rajjou et al., 2008). This carbonylation would result in reduced NADP-ME1 activity, which explains the reduced germination rate observed after seed aging. Thus, protection of NADP-ME1 against ROS attack seems to be an important feature of Arabidopsis seed longevity. NADP-ME1 was not detected as *de novo* synthesized during the time course of seed germination (Galland et al., 2014). Therefore the stored reservoir of NADP-ME1 in the dry seed probably serves to protect the embryo from excessive oxidative pressure that accompanies the increased respiratory metabolism during seed imbibition.

In conclusion, loss-of-function of NADP-ME1 negatively affects seed germination after aging. NADP-ME1 protein is a target of carbonylation during seed aging (Rajjou et al., 2008), and the low abundance of this protein in aged seeds compared to after-ripened seeds (Nguyen et al., 2012), strongly suggests a key role of NADP-ME1 in the germination of aged seeds. The possible role of NADP-ME1 in germinating aged and non-aged seedlings is schematically presented in Fig. 7.

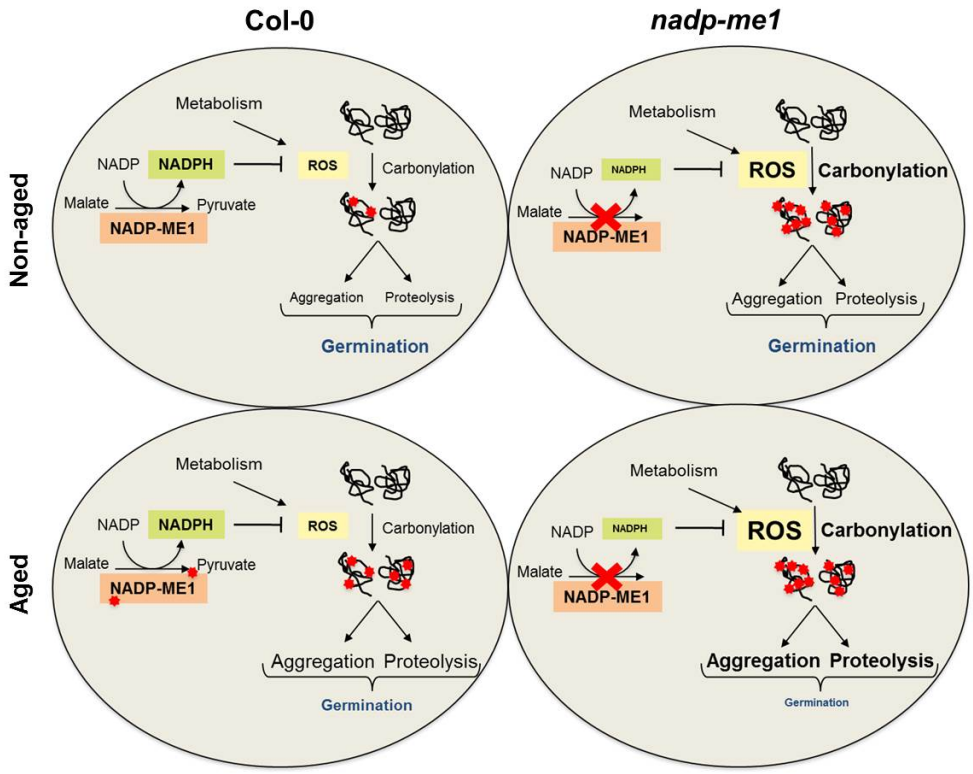

Figure 7. Schematic representation of the (putative) role of NADP-ME1 in relation to seed aging. In non-aged Col-0 imbibed seeds NADPH is produced upon the conversion of malate to pyruvate by NADP-ME1. NADPH provides reducing power for the maintenance of a range of ROS levels that ensure a required level of protein carbonylation (indicated by the red stars) needed for a successful germination. In aged Col-0 and *nadp-me1* seeds there is a reduced availability of reducing power due to reduce amounts (in NADP-ME1 in Col-0) or lack of NADP-ME1 (in *nadp-me1*). As a result, ROS accumulate and cause carbonylation of proteins that are degraded by the proteolysis. Because of this the germination capacity of the seeds is dramatically reduced. The reduction of germination is more severe in *nadp-me1* seeds.

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SUPPLEMENTARY FIGURES

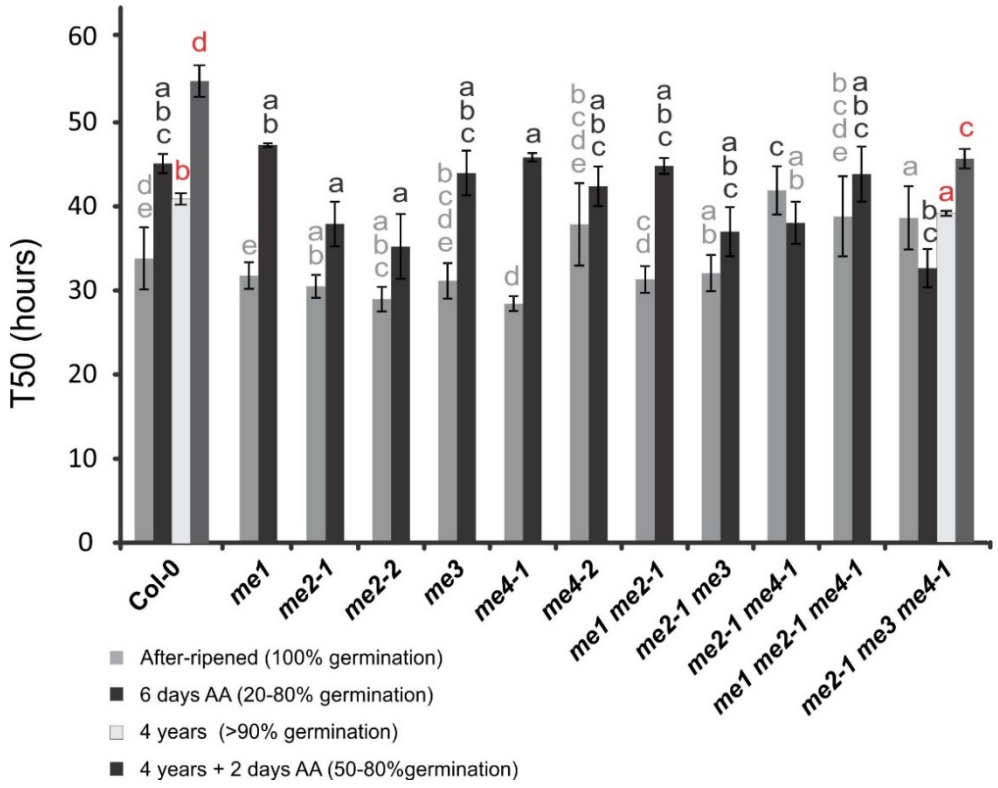

Fig S1. Germination rates for seeds with different storage times. Time that is required to reach 50 percent germination (T50) of fully after-ripened seeds that germinate 100% (dark grey bars) and of seeds after 6 days of accelerated ageing (black bars; T50 values for the total germination that is presented in Figure 1C of the main text) of *me1*, *me2-1*, *me2-2, me3-1*, *me4-1*, *me4-2* their double (*me1 me2-1*; *me2-1 me3* and *me2-1 me4-1*) and triple (*me1 me2-1 me4-1* and *me2-1 me3 me4-1*) mutants. Four years old seeds and four years old seeds + accelerated ageing are seeds of the same seed batch used in Figure 1 but with an additional three years of seed dry storage (total age four years). These seeds were in the accelerated ageing conditions for 2 days. Data only presented for Col-0 and the triple mutant *me2-1 me3 me4-1*, germination curves of this experiment are shown in Supplemental figure 2. Shown are averages of four biological replicates and their SE. Letters indicate significant differences within the treatment, as determined by Tukey HD test ($P < 0.05$). For the four year old seeds the Tukey HD test also allows comparison between the treatments (red letters). Overall ageing (both in dry conditions as well as under accelerated ageing conditions) results in a reduction of the germination rate.

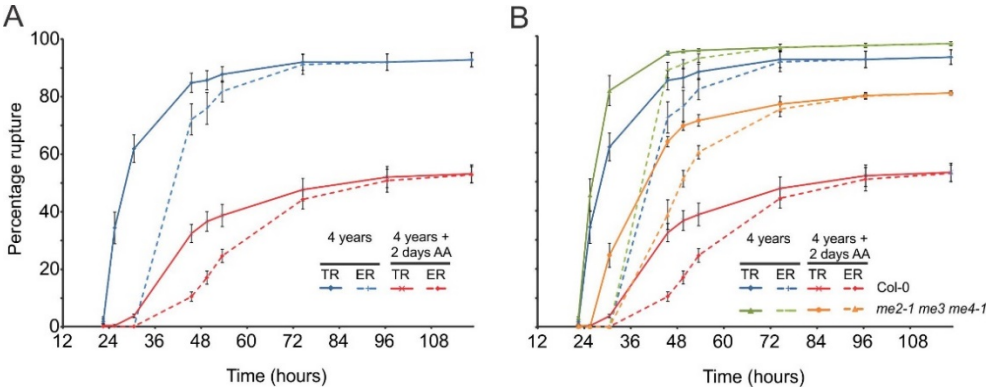

Fig S2. Detailed analyses of testa rupture (TR; solid line) and endosperm rupture (ER; dashed line). This experiment has been performed on the same batch of seeds that was used in Figure 1, however three years later (four years old seeds). A) Germination in wild type Columbia-0 (Col-0). TR as well as ER are delayed in time when comparing four years old seeds to the same seeds that have been accelerated aged (AA) for 2 days. Seeds that do show TR do eventually germinate. B) Germination of Col-0 and the *nadp-me2-1 x nadp-me3 x nadp-me4-1* triple mutant seeds after 4 years of storage and 4 years of storage + AA for 2 days. Averages of four replicates and SE are presented.

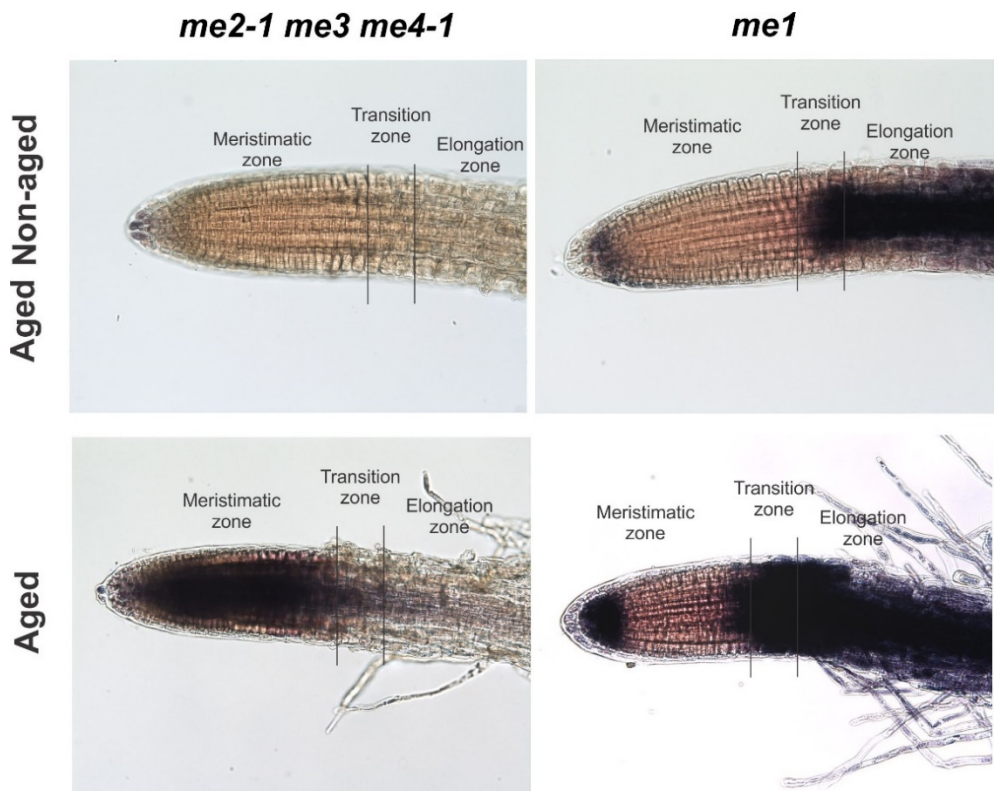

Fig S3. Zoom into the root tip of non-aged and aged seedlings of the *napd-me2-1 nadp-me3 nadp-me4-1* (*me2-1 me3 me4-1*) triple mutant and the *nadp-me1* (*me1*) single mutant. The meristimatic, transition and elongation zone are indicated. These are most clearly visible in the non-aged triple mutant, due to lack of staining. NADP-ME1 activity upon ageing is localized in the meristimatic region seen by the lack of staining in the *me1* mutant aged seed. The activity at the very tip of the root of and in the transition and elongation zone in *me1* is likely due to other MEs. Although their activity seems to be increased upon ageing, they do not affect germination after ageing.

Supplemental table 2. Primers used in this study

Name	Primer	Purpose
At2s3p promotor attB4	GGGGACAACTTTGTATAGAAAAGTTGGACGCCCTTGAAACCAAATTAACAT	Cloning of At2S3P promoter in gateway vector
At2s3p promotor attB1r	GGGGACTGCTTTTTTGTACAAACTTGTGTTTTGCTATTTGTGTATGTTTTCT	Cloning of At2S3P promoter in gateway vector
overlap-Alig-NADP-ME1-Forward	AGAAAACATACACAAATAGCAAAACATGGAGAAAGTGACCAACTCA	Cloning of ME1 coding region
Malic1-Reverse	TCAACGGTAGAGACGGTATGT	Cloning of ME1 coding region
Overlap-NADP-ME1-terminator-Forward	ACATACCGTCTCTACCGTTGAAAGTTGAAACTCAAGAGTCT	Cloning of ME1 terminator
NotI-NADP-ME1-terminator-Reverse	GCGGCCGCTTCTCAGTGGTGTAGGTTAG	Cloning of ME1 terminator
seed-specific-promoter-Forward	CGCCCTTGAAACCAAATTAACAT	PCR to retrieve amplicon of At2S3P from gateway construct
NADP-ME1-ATG-Reverse	TGAGTTGGTCACTTTCTCCAT	PCR to retrieve amplicon of At2S3P from gateway construct

Chapter 5— A role for *Allantoate Amidohydrolase* (*AtAAH*) in the Germination *of Arabidopsis thaliana* Seeds

Farzaneh Yazdanpanah, Henk W. M. Hilhorst, Leónie Bentsink

ABSTRACT

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 of 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. Recently, we showed that *ALLANTOATE AMIDOHYDROLASE* (*AtAAH*) transcripts are up-regulated in imbibed dormant seeds compared to after-ripened seeds. AAH is an enzyme in the uric acid catabolic pathway which releases two molecules of ammonia and CO_2. 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 urea, whereas energy related metabolites and several amino acids are lower upon seed imbibition in comparison to Columbia-0. *AtAAH* expression could be detected during the early stages of seed development with a transient increase around eight days after pollination. *AAH* expression is the highest in mature pollen. The application of exogenous nitrogen can partly complement the higher dormancy phenotype of the *Ataah* mutant seeds. Based on these findings we conclude that a defective AtAAH leads to a reduction in nitrogen (ammonia) release in freshly harvested seeds, this causes a block of germination which can be overcome by seed after-ripening or by the application of exogenous nitrate.

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INTRODUCTION

Seed dormancy is an adaptive trait that regulates the timing of seed germination. Environmental factors including light (i.e. light quality, 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 favour nitrate accumulation in mother plants and seeds lead to lower seed dormancy levels (Alboresi et al., 2005). Seed dry storage, also referred to as after-ripening, leads to a reduction of seed dormancy levels. After-ripening can also be seen 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). 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 plants that are adapted to higher pH and more aerobic soils prefer nitrate (for review see Maathuis, 2009). If nitrate is absorbed, it is first reduced to nitrite and then to ammonium for incorporation into amino acids, nucleic acids, and chlorophyll (Smil, 2000). Another source of nitrogen for expanding leaves at the vegetative stage as well as for seeds at the reproductive stage is the nitrogen that is remobilized from senescing leaves (Malagoli et al., 2005, Diaz et al., 2008, Lemaître et al., 2008). Plants, fungi, and bacteria have metabolic pathways for the re-use of the nitrogen present in pyrimidine and purine bases (Tipton, 2006, Vogels and Van der Drift, 1976). Through a series of enzymatic steps, purines are oxidized to allantoin and allantoate, compounds that have a favourable N:C ratio to serve as nitrogen transport and storage. In plants, the first step of ureide metabolism occurs in the cytoplasm and leads to the production of oxopurines, such as hypoxanthine, xanthine, and uric acid. The opening of the uric acid ring and elimination of carbon dioxide yields S-allantoin through a three-step peroxisomal pathway (Ramazzina, 2006). Hydrolysis of S-allantoin by ALLANTOINASE (ALN) in the endoplasmic reticulum yields allantoate (Hanks et al., 1981) and the breakdown of allantoate produces usable nitrogen for subsequent anabolic reactions. This can occur

by two different pathways either involving ALLANTOATE AMIDOHYDROLASE (AAH) that produces ammonia or ALLANTOICASE (ALLC) that forms urea (Figure 1A). Arabidopsis plants defective in AAH prevent the remobilization of purine nitrogen, as a result the Ataah T-DNA mutant is unable to grow on allantoin as sole 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 (DOGNILs). Seeds of these lines that had increased dormancy levels, induced by growing plants in low nitrate and low temperature conditions, have reduced seed allantoin and urea contents when compared to seeds developed in control conditions (He et al., 2015). Transcriptome analyses on the same seeds showed an up-regulation of *ALN* gene expression (He et al., 2015) which is probably a feedback reaction caused by the lack of ammonia. Moreover, it is known that abscisic acid (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 abscisic acid (ABA) production and enhanced abiotic stress tolerance (Watanabe et al., 2010, Watanabe et al., 2014a, Watanabe et al., 2014b). Furthermore, Ataln mutant seeds displayed high 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 an increased seed dormancy level compared to wild type Columbia-0 (Col-0). This dormancy could be partly compensated by the application of exogenous nitrogen, either during the growth of the mother plant or during imbibition of the seeds.

MATERIALS AND METHODS

Plant material

Arabidopsis thaliana wild accession Columbia Col-0) and homozygous *Ataah* T-DNA insertion mutants (SALK_112631; At4g20070) were used for these analyses.

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).

Plant 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 growth chamber 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 (Table S1) and watered three times per week. For nitrate treated plants, upon the start of flowering, the nitrate regime of plants was changed to 0, 5 and 20 mM resulted in corresponding (low in N0, standard in N5 and high in N20) nitrate levels in the mature seeds, for each condition four biological replicates containing four plants per replicate.

Germination Assays

Germination tests to monitor the release of seed dormancy were performed as described by Joosen et al. (2010) with small adjustments. In short, at several intervals during seed dry storage, until all seed batches had reached 100 percent germination, aliquots of 50 to 100 seeds of each genotype were evenly sown on a filter paper soaked with 0.7 ml 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 one week in a climate chamber, continuously illuminated by 38-W Philips TL84 fluorescent tubes at 8 $W.m^{-2}$ in continuous light at 22°C. After that, the total number and the number of germinated seeds was scored and percentages calculated.

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 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. Stress conditions were: osmotic stress (-1 MPa mannitol; Sigma-Aldrich), salt stress (130 mM NaCl; Sigma-Aldrich), ABA stress (0.15 µM 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 ZnSO4 solution (40°C, 85% relative humidity) in a closed tank for 5 days (ISTA., 2012). After incubation the seeds were taken out and germinated on demineralized water as described before.

Nitrate determinations

To measure nitrate content, 5 mg of seeds were boiled at 100°C for 15 minutes in 0.5 ml 0.5 M HCl and 50 mg l-1 trans-aconitate (internal standard). After centrifuging for 2 minutes at 13000 rpm, 200 µl 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 500 mM NaOH followed by 20-35 min 5 mM NaOH. Flow rates were 1 ml min-1 throughout the run. Contaminating anions in the eluents were removed using 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. External standard of nitrate was NaNO3.

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: 3-5 mg freshly harvested seeds were used for the extraction. Lysis was performed using 315 µL buffer RAP + 35µL Plant RNA Isolation Aid (Ambion: AM9690) + 3.5µL β-Mercaptoethanol (Sigma: M6250). Final RNA was eluted in 40µL RNAse free water. Quality and concentrations were measured by loading 2µL 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 RNA using the iScript cDNA Synthesis Kit (Bio-Rad: 170-8890) according to the manufacturer's protocol. cDNA was diluted 10 times with sterile milliQ water. For each sample 2.5µL cDNA, 5µL iQ SYBR green supermix (Bio-Rad: 172-5125) and 0.5µL primer mix (10µL work solution) were added and

supplemented with water to 10µL. RT-qPCR was performed on a CFX connect (Bio-Rad).

Sequences of the primers of the target genes are presented in supplemental Table 2. Expression of the genes was normalized by the expression of two reference genes that are stably expressed in dry seeds: At4g12590 and At4g34270 (Dekkers et al., 2012). 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 dry mature seeds of the *Ataah* mutant and Col-0 wild type 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 five mg of seeds pre-cooled in liquid nitrogen, was homogenized in 2 ml tubes with 2 iron balls (2.5 mm) using a micro dismembrator (Mo Bio Laboratory). 233 µl methanol/chloroform (4:3) was added, together with 50 µl standard (0.13 mg ml-1 ribitol) and mixed thoroughly. After 10 minutes of sonication 66 µl MQ water was added to the mixture followed by vortexing and centrifugation (5 min., 15000 rpm). The methanol phase was collected in a glass vial. 166 µl methanol/ chloroform (1:1) was added to the remaining organic phase and kept on ice for 10 min. 66 µl MQ water was added followed by vortexing and centrifugation (5 min., 15000 rpm). Again the methanol phase was collected and mixed with previously collected phase. 60 µl was dried overnight using a speedvac (room temperature, Savant SPD121). Dried samples were derivatized online as described by Lisec et al. (Lisec et al., 2006) using a Combi PAL autosampler (CTC Analytics). The derivatized samples were analysed by a GC-TOF-MS system consisting of an Optic 3 high-performance injector (ATAS) and and Agilent 6890 gas chromatograph (Agilent Technologies) coupled to a Pegasus III time-of-flight mass spectrometer (Leco Instruments). 2 µl of each sample was introduced to the injector. The details of the GC-TOF-MS method as described by (Carreno-Quintero et al., 2012) with some minor modifications. Detector voltage was set at 1650V.

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 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).

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 (Figure 1B). A possible role for *AtAAH* during seed development is underlined by its high expression in pollen (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) (Supplemental Figure 1).

The *Ataah* mutant displays increased seed dormancy, expressed as after-ripening requirement (DSDS50), and a higher sensitivity to salt (NaCl) and mannitol; however it is more tolerant to ABA (Figure 1CD). The mutation did not affect seed longevity (germination after artificial aging). The similar pattern between salt and mannitol indicates that the inhibition of the germination is likely an osmotic effect.

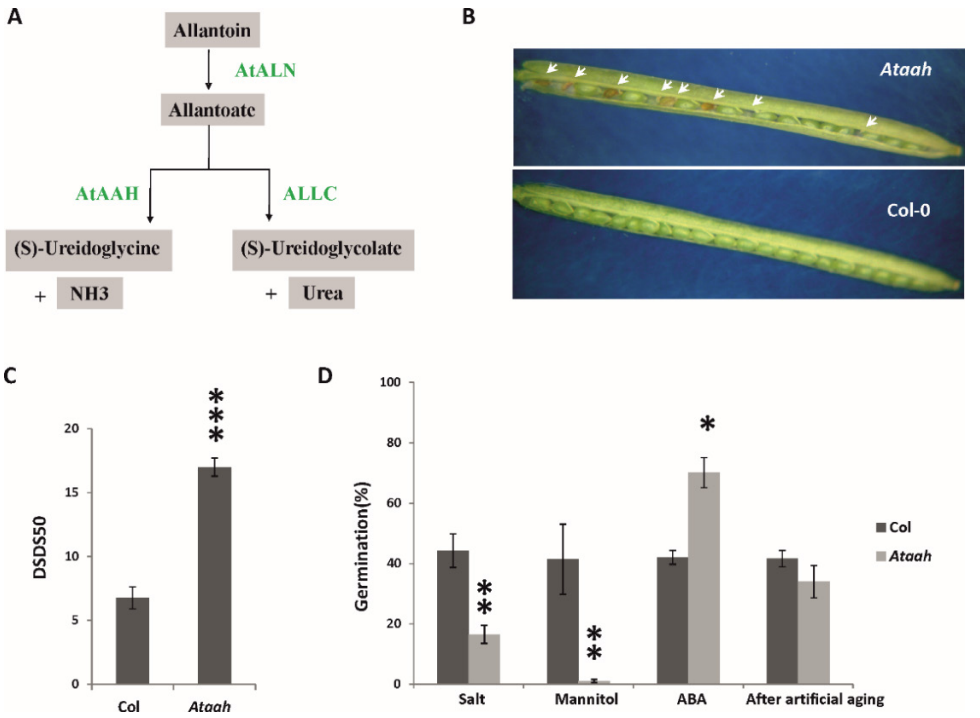

Figure 1. The S-Allantoin degradation pathway and the effect of a defective AtAAH enzyme on Arabidopsis seeds phenotypes. A) Schematic presentation of S-Allantoin degradation in plants. Conversions in the pathway and the responsible enzymes are indicated. B) Open silique of the *Ataah* mutant in comparison to Columbia-0 (Col-0). The mutant contains several defected 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 Col. D) Seed performance of after-ripened seeds (germination after artificial aging and germination in stress conditions including: salt 130 mM; Mannitol (-1MPa) and ABA(0.15 uM)). Shown are averages of four biological replicates and their SE. Significant differences between *Ataah* and Col-0 are indicated (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$).

Expression of *AtAAH* during plant development

The expression pattern of *AtAAH* over plant development was analysed by qRT-PCR in Columbia (Col-0). In addition to its expression in pollen, the gene is expressed all over plant development, but it is especially high in seedlings, roots and in siliques eight days after pollination (Figure 2A).

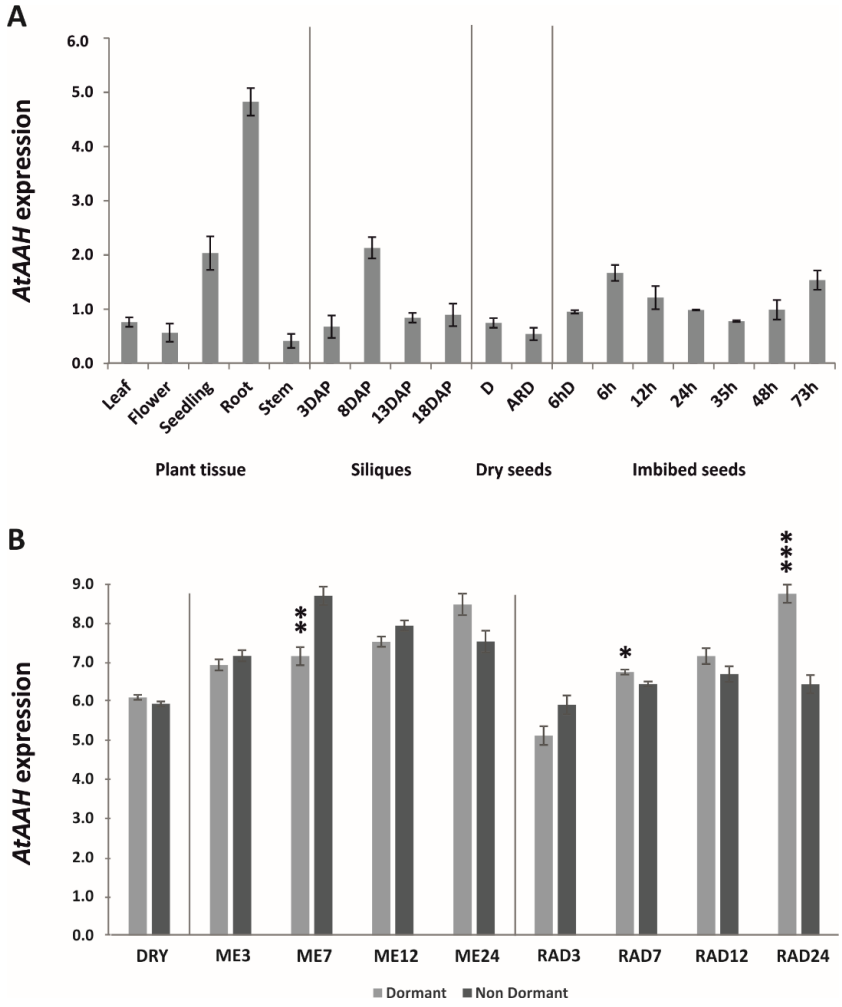

Figure 2. Expression pattern of *ALLANTONATE AMIDOHYDROLASE* (*AtAAH*) during plant development. A) Mean relative expression levels of *AtAAH* measured by qRT-PCR in tissues of the Columbia accession Leaf (L), flower (F), seedling (SE) root (R) stem (ST), in siliques 3, 8, 13 and18 days after pollination (DAP), in dormant dry (D) and 6-hour imbibed seeds (6hD), and across the germination time course in after-ripened dry (ARD) and 6, 12, 24, 35, 48 and 73 hours 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 hours after imbibition. Asterisks indicate significant differences of dormant and non-dormant in *AtAAH* expression (three asterisks, $p < 0.001$; two asterisks, $p < 0.01$; one asterisk, $p < 0.05$).

Earlier we identified AtAAH as a dormancy-up gene, based on the higher expression in dormant compared to after-ripened seeds (Yazdanpanah et al., 2017). To investigate how the higher expression in dormant versus after-ripened seeds matches the more dormant phenotype of the KO mutant, the expression pattern of *AtAAH* was analysed in Cvi 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 less in non-dormant seeds (Figure 2B).

Metabolic changes in the *Ataah* loss of function mutant

GC-MS metabolite profiling on 24 hour 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). Furthermore, contents of the amino acids serine, threonine, isoleucine and glycine were strongly reduced. There was also a reduction in the organic acids malate, fumarate, citrate and succinate in the mutant as compared to wild type (Figure 3). Allantoate was not detected by the analysis.

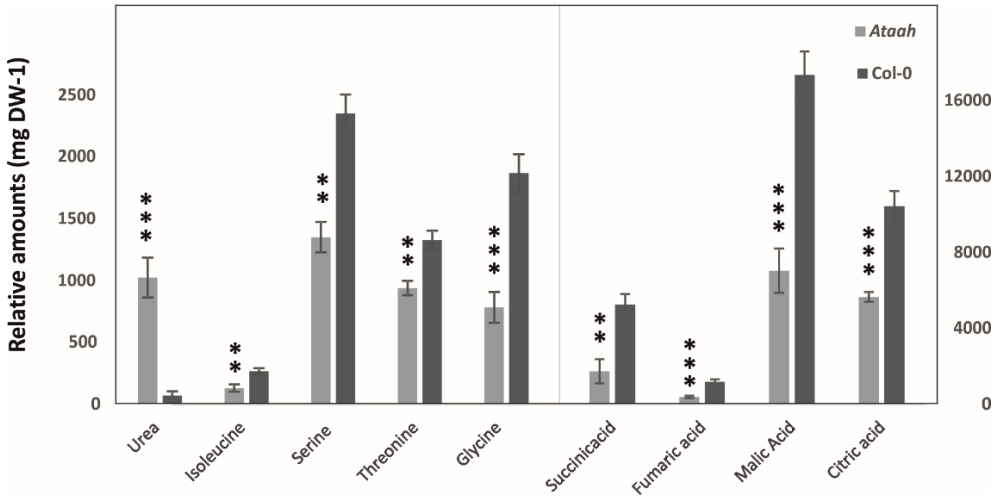

Figure 3. Metabolite differences between the *Ataah* mutant and wild type Col-0. Relative metabolite amounts (mg DW^{-1}) in *Ataah* and Col-0. Metabolite levels were measured by GC-MS (see Material and Methods for details). Asterisks indicate significant differences relative to the respective wild type (three asterisks, $P < 0.001$and two asterisks, $P < 0.01$).

Exogenous 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 ammonia, we applied exogenous nitrate to the mother plant during seed development and to the seeds during seed imbibition. Nitrate assimilation of parent plants during seed maturation affect the accumulation of nitrate in seeds. Therefore different nitrate 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 wild type (supplemental Figure 2A). Treatment of the *Ataah* mutant with the highest nitrate level largely overcame the increased dormancy of the mutant compared to that of the wild type Col-0 (Figure 4A), but it could not prevent the seed abortion in the mutant. Similar results were obtained for the germination in salt and mannitol (Figure 4BC). The application of the highest nitrate concentration (20 mM) did completely abolish the difference between the wild type and mutant when germinated in mannitol (Figure 4C). The germination of the mutant in ABA was not affected by the application of nitrate (Figure 4D). Also the seed ABA levels did not differ among seeds that were grown in the different nitrate regimes (Supplemental figure 2B).

Freshly harvested (five days after harvest) dry seeds that were developed under the three nitrate regimes have been imbibed in three different nitrogen sources (nitrate, ammonium). For all genotypes, germination of seeds treated with KNO3 was higher ($P < 0.05$) as compared to the germination in water (Figure 5A). Thus, both wild-type and mutant seeds responded to exogenous nitrate, however the mutant response was more obvious. The application of KNO3 rescued the germination of the mutant, as the germination of the mutant with the addition of KNO3 is higher than that of wild type in water for all three nitrogen seed maturation regimes. Imbibition on NH4NO3 and NH4Cl did not affect the germination percentage (Figure 5BC), although NH_4OH likely had a toxic effect considering the fact that the non-germinating seeds turned dark (data not shown).

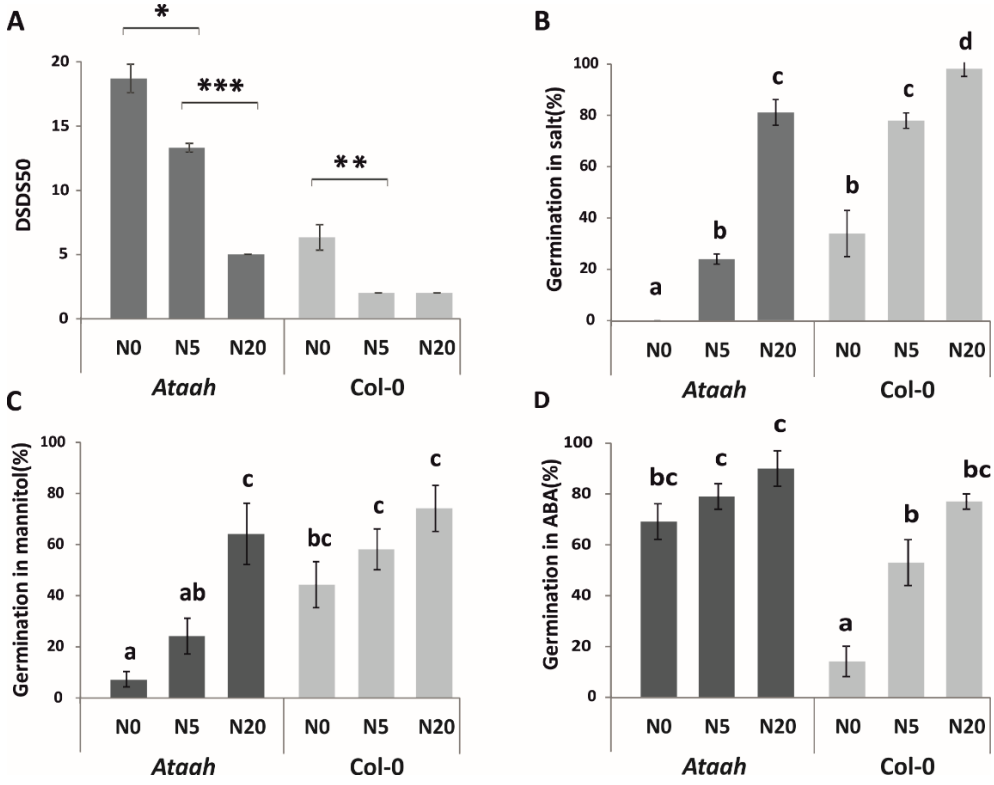

Figure 4. Effect of different nitrate regimes during seed maturation on seed performance. Seed performance of both *Ataah* and Col-0 is presented. A) Dormancy level (DSDS50), B) germination in salt (130 mM NaCl), C) Germination in mannitol (-1 MPa), D) Germination in ABA (0.15 µM) of seeds matured on plants exposed to different nitrate levels (N0, N5 and N20). Shown are averages of four biological replicates and their SE. Asterisks indicate significant differences (three asterisks, $p < 0.001$; two asterisks, $p < 0.01$; one asterisk, $p < 0.05$).

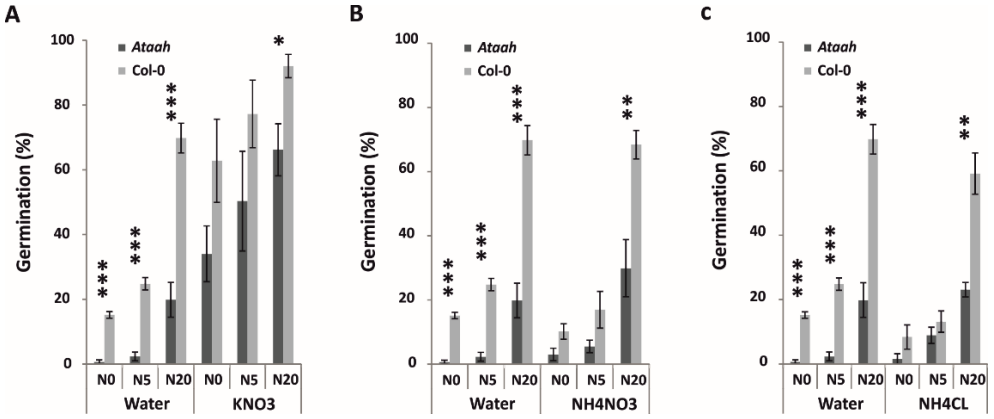

Figure 5. Effect of feeding with nitrogen-containing components during seed imbibition on germination rate of mutant (*Ataah*) and Col-0 seeds matured on plants exposed to different nitrate levels. Germination rate of seeds after feeding with KNO3 (10 mM; A), NH4NO3 (10 mM; B) and NH4Cl (10 mM; C) during 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 *** $P < 0.001$).

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) reverse transcription quantitative PCR (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 (Figure 6A). Further, only *AtALN* and *URE* displayed slightly but significantly higher expression in seeds of the *Ataah* mutant which had developed under N0, as compared to the other nitrate regimes (Figure 6BC). *AtALN* expression in the mutant was also significantly higher at N0 than that in Col-0 at the same concentration (Figure 6B).

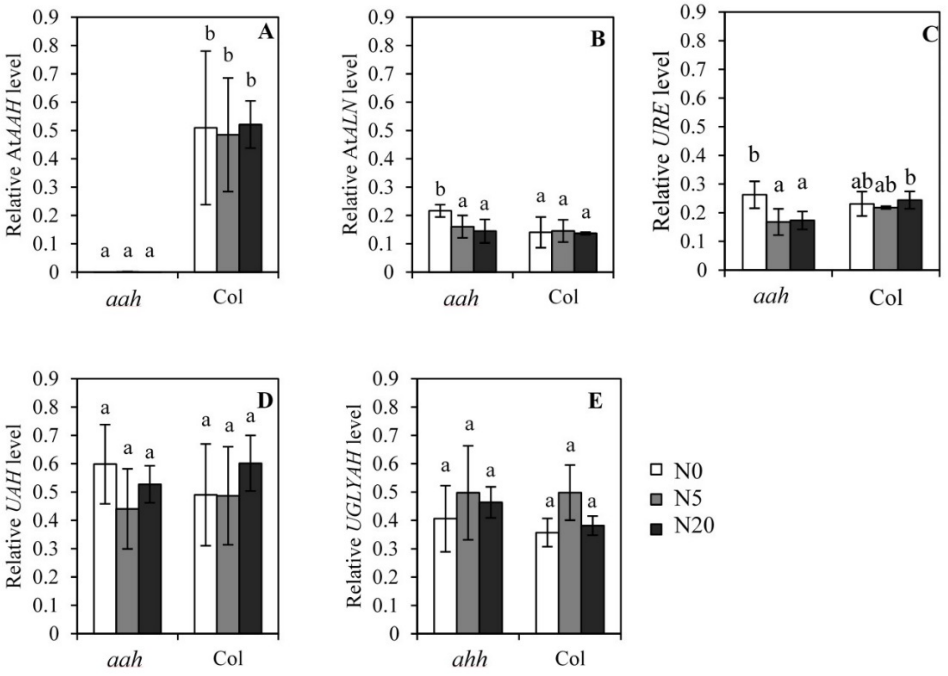

Figure 6. Relative expression of genes in the purine pathway related to allantoate degradation: *AtAAH* (A), *AtALN* (B), *URE* (C), *UAH* (D) and *UGLYAH* (E). Plant material consisted of seeds of Col and the *ataah* mutant matured on plants exposed to different nitrate levels. Means of relative expression level (reference genes At3g25800 and At4g34270) from four replicates are presented. Error bars represent standard errors, and different letters indicate statistical significant differences (Student t-tests; $P < 0.05$).

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 of AtAAH for recycling purine-ring nitrogen has been demonstrated (Werner et al., 2008). In the present study we interpreted the lack of germination in freshly harvested seeds as increased seed dormancy, but actually mutating AtAAH seems to result in nitrogen shortage for germination. During the after-ripening process the germination window widens and therefore ultimately less nitrogen is required for germination (Finch-Savage et al., 2007). *AtAAH* was found as a dormancy-up gene (Yazdanpanah et al.,

2017; Figure 2B), however this does not necessarily mean that this gene is required for dormancy maintenance. More likely its expression in dormant seeds is induced to overcome the block of germination by increasing internal nitrogen resources.

Ataah mutant seeds contain a higher amount of urea compared to Col-0 and, therefore, we hypothesize that the nitrogen requirement of the mutant seeds activates the purine pathway, as AtAAH is missing, allantoate is converted to ureidoglycolate which leads to the production of urea (known as the urea producing pathway) instead of the conversion to uredoglycine which has ammonia as a side product (known as the ammonia producing pathway). To release nitrogen, the ureides must be hydrolysed generating ammonia for re-assimilation into amino acids. Arabidopsis mutants lacking a functional AtAAH are not able to breakdown ureides (Todd and Polacco, 2006). Probably, this is the reason for the lower abundance of the amino acids, serine, threonine, isoleucine and glycine in Ataah seeds (Figure 3), consequently this might inhibit the translation of transcripts that are necessary for germination. Seeds of the Ataah mutant with increased dormancy, have also reduced energy metabolism and TCA cycle activity in comparison with Col-0, as can be concluded from the reduced amounts of malate, fumarate, citrate and succinate in the mutant comparing to wild type (Figure 3).

To test if the higher dormancy of the *Ataah* seeds is indeed the consequence of a defective ammonia production, exogenous nitrate was applied during seed maturation and seed germination. The 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 ABA. Exogenous nitrate did also not affect the lower sensitivity of *Ataah* seeds to ABA. This indicates that the ABA sensitivity in the mutant is not directly related to nitrogen as a resource. Previous research has demonstrated that loss-of function of AtALN resulted in activation of ABA metabolism (Watanabe et al., 2014b). However, ABA measurements in freshly harvested *Ataah* and Col-0 seeds, produced under different nitrate regimes, revealed that this is not the case in the *Ataah* mutant (Figure S1). Together with the germination assays in ABA this suggests that ABA is irrelevant to the dormancy phenotype of the mutant.

Col-0 seed dormancy and germination under stress was only affected by a reduced nitrate (0 mM) content. Apparently, N5 or higher nitrate concentrations are saturating in Col-0. Among the various nitrogen compounds applied during the imbibition of freshly harvested seeds, only KNO3 stimulated germination. It completely rescued the dormancy phenotype of the mutant, indicating that a defect in *AtAAH* functioning during seed germination and the resulted lack of ammonia can be overcome by exogenous nitrate.

Two pathways are presently known for allantoate degradation; the first is catalyzed by ALLANTOICASE (ALLANTOATE AMIDINOHYDROLASE, ALLC), the second by ALLANTOATE AMIDOHYDROLASE (AAH). Both enzymes are present in soybean germplasm and different cultivars may employ one pathway exclusively or both (Vadez and Sinclair, 2000). There are several reports of the Ataah mutant accumulating high levels of the substrate allantoate (Todd and Polacco, 2006, Werner et al., 2008, Werner et al., 2013), indicating that AtAAH is the main enzyme involved in allantoate degradation. Our metabolite profiling identified higher urea contents in *Ataah* seeds, which indirectly provides evidence for increased allantoicase activity in imbibed dormant *Ataah* seeds.

It is known that ammonia inhibits both uptake of urea and urease activity (del Mar Dobao et al., 1993). Thus, it was speculated that ammonia deficiency in the mutant activates URE to provide ammonia from accumulated urea. Since the rate constant of URE is lower than AtAAH (Yata et al., 2015) this may result in the higher inhibition of germination in freshly harvested *Ataah* dormant seed compared to those of Col-0.

Alboresi and co-authors (2005) showed that nitrate can act as a signal molecule in the control of seed dormancy. Seeds use nitrate sensing as a gap in the canopy detection mechanism, as nitrate levels inform about the presence of competing plants that deplete soil nitrate (PONS, 1989). In the deeply dormant Arabidopsis Cvi ecotype, nitrate can substitute for the long period of dry storage (7 – 12 months after ripening) or several days of cold stratification required for dormancy release (Finch-Savage et al., 2007). Results of exogenous nitrate application to seeds of wild-type and nitrate reductase (NR) deficient Arabidopsis mutant seeds support a role of nitrate as a signal during germination that induce expression of the ABA catabolic gene *CYP707A2* (Ali-Rachedi et al., 2004, Alboresi et al., 2005, Matakiadis et al., 2009). Although we

cannot exclude a role for nitrate signalling our data clearly revealed a nitrate nutritional effect on seed germination. The reduction in amino acids content in the mutant supports this idea. Furthermore, feeding germinating seeds matured on three nitrate levels, with KNO3 clearly promoted the germination in mutant therefore, abolished or moderated the germination rate differences of mutant and Col-0 found of their germination in water. These results confirm that AtAAH activity is required for releasing the inhibition of germination.

In developing seeds ureides must be converted so that its metabolic intermediates can be incorporated in nitrogen-containing compounds like amino acids. nitrogen utilization of allantoate requires its two ureido groups to be converted to ammonia (Lamberto, 2010). The expression profile of *AtAAH* obtained using the Arabidopsis eFP browser shows that *AtAAH* transcripts are present in early seed development (Supplemental figure 1). This is consistent with our expression analysis (Figure 2A) which demonstrates transcript abundance of the gene during the early stages of seed development with a transient increase around eight days after pollination. However, by far the highest transcript abundance was found in mature pollen (Supplemental figure 1). It is possible that the lack of this enzyme during early embryogenesis causes abortion of *Ataah* mutant seeds. However, concerning the fact that siliques of mutated plants contain both normal and aborted seeds it is more likely that abortion is a result of reduced efficiency of the pollen in fertilization rather than being the consequence of a defect in embryogenesis of the mutant seeds.

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SUPPLEMENTARY DATA

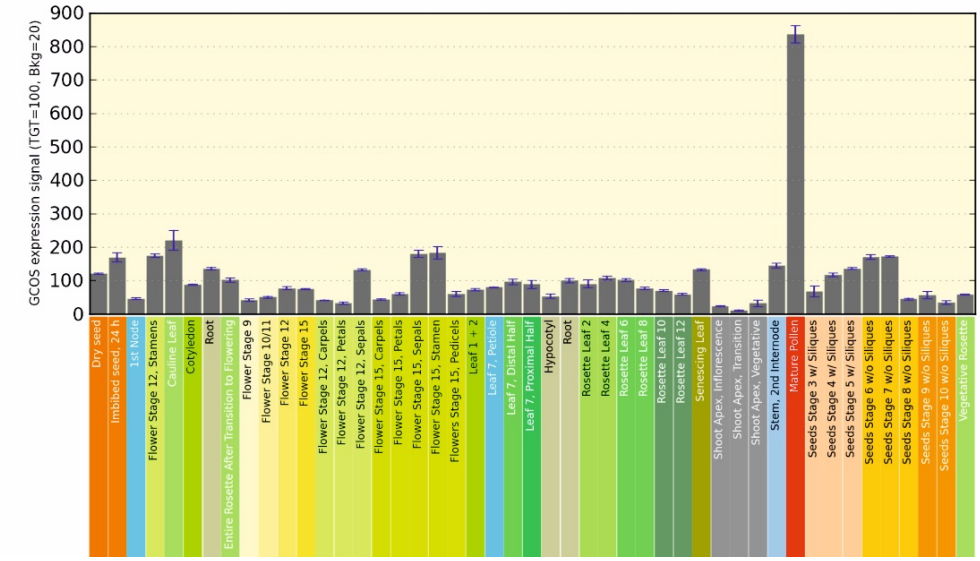

Figure S1. Expression of *AtAAH* among various plant tissues and developmental periods. Note the strong expression in mature pollen as viewed with the Arabidopsis eFP Browser.

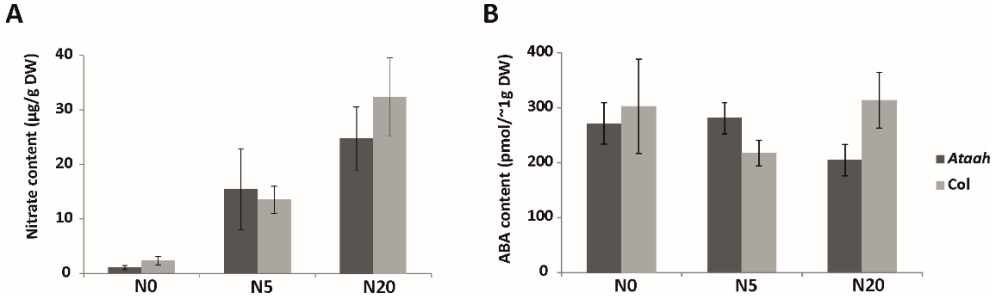

Figure S2. Nitrate and ABA content of seeds produced under different nitrate regimes. A) Nitrate content (mg/l) and B) ABA concentration (pmol/g DW) of freshly harvested seeds of allantoate amidohydrolase mutant (*Ataah*) and Col-0 after seed maturation on plants exposed to different nitrate regimes (N0, N5 and N20). DW, Dry weight.

Table S1. 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	Mo
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

Table S2. Primer pairs used for qrtPCR analyses

Gene	**Primer sequence (5'-3')**	
AtAAH/AT4G20070	F: CATTGAACGAAAGCACGATG	R: TCCTGCTCCACTCATTAGCA
AtALN/AT4G04955	F: AGCAAAAGGCAAAGGAGACA	R: CTGGAATCTCTTCGGCTGAG
UGLYAH/AT4G17050	F: ATCGCTTGGGTGATAACTGG	R: CAGCATACCATTGAGGGACA
URE/AT1G67550	F: GCATTTCGTGGGAGAACAAT	R: TGGTTGATGACGGGAGTACA
UAH/AT5G43600	F: ATCTGCCTGCTGTAGCCACT	R: AAACACCGACAACACCATCA

Chapter6— General Discussion

Farzaneh Yazdanpanah

A major problem of seed improvement programs is that genes with a known function for seed quality aspects are limited in domesticated species. Therefore, new candidate genes might be useful for breeders. Seed biologists have employed a variety of approaches to identify genes that contribute to a better seed performance, among which combining knowledge of quantitative trait loci (QTL) (covered in near isogenic lines (NILs)) and transcriptome analyses is considered an insightful approach (Wang et al., 2016). Accordingly, in this thesis, comparative microarray analyses have been performed on freshly harvested (dormant) and after-ripened (AR; non-dormant) 24-hour imbibed seeds of four different *DELAY OF GERMINATION* near isogenic lines (*DOG*NILs) and the Landsberg *erecta* (L*er*) wild type. These genotypes have varying levels of primary dormancy. This analysis led to the identification of the genes differentially expressed in all genotypes tested (46 and 25 genes for the dormancy-up and after-ripened-up set, respectively). Mutating these genes in the model plant *Arabidopsis thaliana* resulted in several dormancy and other germination-associated phenotypes (**Chapter 2**). Since, candidate genes in this thesis were identified in multi-NIL transcriptomic analysis, they are most probably conserved in the NILs. Therefore the chance of finding a germination phenotype by reverse genetics is much higher as compared to a situation in which only the expression profile of a single genotype is examined.
In this thesis we identified genes that are implicated in maintenance of dormancy, in germination and in other traits related to seed performance. For 47 genes a homozygous knock-out mutant was selected and their germination performance investigated. Among these mutants, 25 lines showed a phenotype for at least one seed performance trait. Since a functional analysis for all identified genes would require too much time, I selected three genes to obtain a deeper insight into their molecular mechanisms, based on the seed performance phenotype represented by knock-out mutations of these genes (**Chapters 3, 4 and 5**).

In **Chapter 3,** I performed a physiological analysis of *FAST GERMINATING SEEDS* (*FGS*), a novel gene whose lack of function results in seeds with reduced dormancy, faster and more synchronized germination and reduced sensitivity to abiotic stresses, without affecting seed storability. *FGS* has been annotated to encode a protein with unknown function. Several strategies have been pursued for the further analysis of this gene. A cellular approach revealed that FGS is localized

in protein storage vacuoles (PSVs). Reciprocal crosses indicated that FGS controls seed germination through the maternal tissue. Furthermore, proteome and metabolite analyses of the *fgs* mutant showed a reduced abundance of seed storage related proteins in dormant dry seeds and quicker release of amino acids in germinating seeds in comparison with wild type (Col-0). Taken together, these findings suggest that FGS has a role in seed storage protein mobilization.

NADP-MALIC ENZYME 1 (*NADP-ME1*) was another gene that I chose to study (**Chapter 4**), especially because of the reduced seed longevity phenotype observed in its loss-of-function mutant. NADP-ME1 is one of four Arabidopsis NADP-ME homologs that catalyses the oxidative decarboxylation of malate to yield pyruvate, CO2, and NADPH. We hypothesized that NADP-ME1 is involved in maintaining the cellular redox homeostasis through the production of NADPH during germination. This hypothesis was supported by higher levels of protein carbonylation in nadp-me1 seeds than those of the wild type. Furthermore, a relative increase in NADP-ME1 expression was observed in imbibed aged- as compared to non-aged seeds. In line with transcriptional increases also NADP-ME1 enzyme activity increased during the germination of aged wild type seeds. This again suggests a key role of NADP-ME1 in the germination of aged seeds.

In the last research chapter, I have studied the possible role of *ALLANTOATE AMIDOHYDROLASE* (*AtAAH*) in the germination of *Arabidopsis thaliana* seeds (**Chapter 5**). AtAAH is an enzyme in the final stage of the purine catabolic pathway that converts allantoate to ureidoglycine, ammonia and CO2 (Werner et al., 2008). Seeds of the loss of function *Ataah* mutant showed an increased seed dormancy level compared to wild type (Col-0). Considering the negative correlation of nitrogen with seed dormancy (Alboresi et al., 2005) and the key role of AtAAH for recycling purine-ring nitrogen (Werner et al., 2008), we proposed that a lack of ammonia prevents seed germination in the Ataah mutant. I tested this hypothesis by applying exogenous nitrogen to the mother plant during seed development and to the seeds during seed imbibition. Both partially complemented the higher dormancy phenotype of the mutant seeds. Based on these findings we conclude that a defective AtAAH leads to a reduction in nitrogen (ammonia) release in freshly harvested seeds. This causes a block of germination which can be overcome by seed after-ripening or by the application of exogenous nitrate.

Knowledge transfer to crops

The enhancement of seed performance is a main objective of the agricultural sector and in particular seed- and breeding companies to ensure a reliable and successful establishment of crops for farmers and, as a result, maintain the competitive position of these companies in agricultural markets. Many seed companies are dependent on enhancement methods for seed quality, such as seed priming which is a pre-sowing treatment that involves the controlled hydration of seeds, sufficient to initiate pre-germinative metabolic events, but insufficient to allow radical emergence (Heydecker, 1973; Bradford, 1986). Priming treatments are used to enhance percentage and speed of germination and synchronize the germination of individual seeds. Although seed priming is a commercially successful practice for obtaining better germination and field performance, its influence on crop production and yield has not been always confirmed. For example, optimal priming conditions may differ among cultivars and even seed lots of a given species (Khan and Burrio, 2015). Furthermore, the benefit of priming may be lost during seed storage or in field experiments. Nevertheless, an important practical issue of seed priming is the strong reduction in longevity that limits its application for many species. In order to solve this, a better understanding of molecular, biochemical, and physiological mechanisms regulating seed dormancy, germination and deterioration is required. This knowledge will allow targeted breeding for seed performance.

I anticipate that my work provides leads for smart breeding to genetically optimize and improve the vigour and performance of the seeds. For example, among the various phenotypes identified, germination phenotypes represented by the *fgs* mutant resemble the requirements for vigorous seeds in an agricultural context, i.e. seeds with reduced dormancy and therefore, higher rate of germination, also quick and synchronised germination and less sensitivity to NaCl stress and osmotic stress caused by mannitol (**Chapter 3**).

From an ecological point of view, seed dormancy is an advantageous adaptation to a range of natural conditions. Therefore, seeds of many species are equipped with dormancy mechanisms that enable them to spread germination over time and place, in various environmental conditions (Cohen, 1966; Footitt et al., 2011). Moreover, within species and even within a genotype, seeds normally germinate in

the same season of the year, but usually do not germinate uniformly. Therefore, seed dormancy serves as an adaptive response that buffers against changing environmental conditions, contributing to the survival of individuals, as well as plant communities (Seger, 1987; Vidigal et al., 2016). However, when conditions are right for agronomic productivity, dormancy is not a prerequisite except to avoid pre-harvest sprouting. For this reason, during domestication most crop species have been selected for low dormancy. Low dormancy and faster plant establishment extend the growth period and may contribute to a total increase in yield (Rashid et al., 2004). It may also help to increase plant competition over weeds (Jalali and Salehi, 2013). Despite the agricultural benefits of rapid germination, during domestication in some cases, the selection pressure against dormancy may have gone too far, resulting in a condition known as pre-harvest sprouting and, consequently. substantial economic losses to many crops, in particular grain crops (Clarke et al., 2005; Rodríguez et al., 2015). This is not a problem in varieties with strongly dormant grains. However, high levels of dormancy may also be problematic, because the grains then need a period of dry storage or special treatment in order to break dormancy (Finch-Savage and Leubner-Metzger, 2006). Moreover, slow and uneven germination prevent efficient space usage, especially in greenhouses where space is limited. It also has other financial consequences through wasted planting materials, higher cost of human labour, and reduced quality resulting from variation of the product. Therefore, not only the percentage of germination, but also the speed and uniformity of germination are important in many crops. Nevertheless, low dormancy does not necessarily lead to fast and uniform germination. Seeds with more uniform germination are more synchronized in the germination process and have small variability in relation to the mean germination time (Heydecker, 1973; Derek Bewley and Black, 1994), while seeds with low dormancy need a short dry after-ripening time to obtain germination capacity upon imbibition and may not have uniform germination. Therefore, the identification of mutants that have both characteristics, namely low seed dormancy and uniform germination may prove valuable; fgs is an example of such a mutant (**Chapter 3**).

Regarding crops with pre-harvest sprouting problems, the timing of dormancy release is an important goal for breeders globally. Breeding programs in these crops

aimed at producing seeds that remain dormant before shedding (harvest), but progress to germination and seedling emergence with a minimal delay following sowing (reviewed in Benech-Arnold and Sanchez, 2004; Rodríguez et al., 2015). However, in many cultivated species, such as cereals, molecular genetics and genomics approaches are not highly advanced because these crops contain large and polyploid genomes, and are highly resistant to gene-based technologies. Therefore, as a starting point, the existing molecular genetic information from studies in model species like Arabidopsis have started to be translated into cereal research, especially from a dormancy point of view (i.e. pre-harvest sprouting phenotype), because the regulation of dormancy has been shown to be conserved over species (Wilkinson et al., 2002). For example, *VIVIPAROUS 1* and its homolog *ABSCISIC ACID INSENSITIVE 3*, which play a role in the regulation of dormancy, are conserved among many plant species including Arabidopsis, maize and wheat (Koornneef et al., 1984; McCarty, 1995; McKibbin et al., 2002). In addition, *DELAY OF GERMINATION 1*, another key regulator of seed dormancy that was first identified in Arabidopsis has been found in *Lepidium* species (Graeber et al., 2012) as well as in a number of monocots, such as wheat, barley and rice (Ashikawa et al., 2010; Sugimoto et al., 2010). Therefore, our work may provide possible candidate genes that have a potential role in the regulation of seed dormancy and pre-harvest sprouting in cereals. For example, the *AtAAH* knock-out in my study resulted in seeds with increased dormancy. This makes it a potential candidate to enhance dormancy and reduce pre-harvest sprouting in a crop such as wheat. Coding sequences of this gene have been found in other plant genomes. Nevertheless, for selecting candidate genes, detailed knowledge of both dormancy and pre-harvest sprouting phenotypes is also required.

Seed longevity is another important trait that contributes to the performance of the seed and is defined as a period of dry storage during which the seed remains viable. Knowledge on the control of seed longevity is necessary for various applications, for example, preservation of genetic diversity in seed banks and breeding for varieties that are storable for a long time. Seed longevity is strongly affected by the environmental condition during storage. These environmental conditions namely, temperature, relative humidity and partial oxygen pressure, have been frequently investigated (Roberts, 1972; Nasreen et al., 2000;

Onyekwelu and Fayose, 2007; Shelar et al., 2008; Pradhan and Badola, 2012). However, less attention has been paid to the mechanisms that regulate the loss of viability. Since access to controlled seed storage facilities is limited in much of the developing world, a better strategy would be to develop crops with enhanced storability through breeding or biotechnological approaches. Mutants with either improved or deteriorated seed storability can be used to study the genetics of seed aging (Tesnier et al., 2002). In this thesis I found reduced seed longevity in mutant seeds deficient in NADP-ME1. Further investigation showed a key role for NADP-ME1 in the germination of aged seeds, probably through protection of seed proteins against oxidative damage. Furthermore, up-regulation of *NADP-ME1* provides proof of the fact that improved seed storability can be achieved through understanding the molecular basis of seed longevity (**Chapter 4**).

When working with T-DNA knock-out lines, it is quite common, to have multiple defects, in addition to that of primary interest (pleiotropic defects). Thus, it is often required to analyse various phenotypic parameters including the ones related to seed to avoid the introduction of undesirable traits along with the selected one (Lee and Zhu, 2010). In this thesis I initially measured the seed dormancy level of the mutants, after which the fully after-ripened seeds were tested for seed longevity and germination under stress, such as salt- and, osmotic stress and abscisic acid (ABA) to obtain a wider view of their seed performance. In addition some of the mutants represented altered plant phenotypes that should also be taken in to account since these might affect plant development and as such could also affect the seed traits indirectly. Mutations in dormancy related genes may sometimes be accompanied by pleiotropic effects on other characteristics that are also acquired during seed maturation, including seed longevity and desiccation tolerance (Goldberg et al., 1994; Koornneef and Karssen, 1994). Such mutation is also likely to affect other properties of germination, like germination under stress (i.e. salt or osmotic stress) that often correlates with primary seed dormancy. This correlation can be explained by the fact that dormancy release during seed dry storage (after-ripening), widens the germination window and allows seeds to germinate in conditions that inhibit the germination of freshly harvested seeds (Finch-Savage and Leubner-Metzger, 2006). Moreover, it has been shown recently that salt induces features of a dormancy-like state in seeds of a halophytic relative of

Arabidopsis (Kazachkova et al., 2016). Therefore, germination percentage under salt-induced stress usually reflects the dormancy status of the mutant, as can be seen for the non-dormant *fgs* mutant, which was clearly more tolerant to both salt and osmotic stress (**Chapter 3**) and the *Ataah* mutant which displays elevated dormancy and therefore was more sensitive to those stresses (**Chapter 5**). However, not all the mutants behaved in this way. Alternatively, the study of the new set of mutants presented here with reduced or increased sensitivity to salt should also contribute to a better understanding of salt sensitivity and salt-tolerance mechanisms. Soil salinity is one of the major limitations in many important agricultural areas but the success of breeding programs to solve such agronomic problems is restricted due to the lack of knowledge about the molecular basis of salt tolerance (Chinnusamy and Zhu, 2003).

Finally, as an additional indicator of dormancy, the sensitivity of germination to ABA was also assessed for the mutants. ABA has long been known to play a central role in the induction and maintenance of seed dormancy. Increased ABA sensitivity or ABA accumulation results in increased seed dormancy, while reduced ABA biosynthesis or ABA-insensitivity results in decreased seed dormancy (reviewed by Finkelstein et al., 2008). This plant hormone also plays a central role in regulating plant responses to various environmental stresses (O'Brien and Benková, 2013).

The functional characterization of Arabidopsis candidate genes that were discussed here, likely provides information that is also valid for other plant species. Therefore, one of the desired follow-ups of this study is to use the results for developing crops with improved and stable seed performance, preferably in a wide range of environments. For the translation of our findings to crops of interest, after isolating candidate genes, the crop orthologs must be found. In addition, the natural variation of those orthologs within crop species could be exploited. Thereafter, these crop orthologs could be expressed in Arabidopsis mutant lines to determine whether any of the natural alleles can rescue the mutant phenotype (Ferrier et al., 2011). If orthologous gene functions are validated in the crop, then different strategies such as overexpression, designing synthetic microRNAs, mutagenesis or TILLING and genome-editing tool (CRISPR-Cas9 technology) can be used for up- and down-regulation of the expression (Barrero et al., 2010). In the cases of weak homology or dissimilar function of observed orthologs the focus

should be placed on revealing the underlying mechanisms and networks rather than single gene comparisons (Ferrier et al., 2011).

Variation in amino acid levels in seeds mutated in selected genes: same concern different origin

The levels of free amino acids in seeds vary strongly and change dynamically depending on the seed's developmental and physiological states (Hildebrandt et al., 2015). During seed development and maturation, many amino acids are synthesized and used for the synthesis of storage proteins (Bewley and Black, 1994). Storage proteins are protected against premature breakdown during their formation and deposition, whereas they become completely degradable upon seed germination to provide amino acids for the biosynthesis of the proteins required for seedling establishment (Müntz, 1998). The start of storage protein mobilization is a sign that the protective mechanisms against proteolysis have been overcome. However, protein degradation and mobilization in germinating seeds does not start in all cells and seed tissues simultaneously but follows specific spatial and temporal degradation patterns (Hara and Matsubara, 1980; Bethke et al., 1998; Tiedemann et al., 2000). During seed imbibition, degradation of storage protein begins at the radicle tip and pre-vascular strands, but during germination it mainly occurs in the cotyledons (Müntz, 2007). The dependence of germination on storage protein degradation is well supported by the finding that inhibition of germination by ABA in Arabidopsis occurs by preventing storage protein degradation (Garciarrubio et al., 1997; Weitbrecht et al., 2011) and not by a deficiency in lipid mobilization (Penfield et al., 2005).

For the seeds mutated in *FGS*, the significant decrease in the storage related proteins and the increase in total free amino acid content during germination in comparison to Col-0 wild type (**Chapter 3**), suggests that amino acids are provided by the mobilization of stored proteins. It also shows that germinating *fgs* seeds are more advanced in the mobilization of proteins and this led to their fast and uniform germination.

Despite the importance of storage proteins in providing primary amino acids for seed germination, the composition of reserve amino acids is radically different from the one required for cytoplasmic protein formation. Thus, there is a crucial need for

inter-conversion and de novo synthesis of amino acids (Lea and Joy, 1983). The biosynthesis of new amino acids requires inorganic nitrogen (ammonium) to be assimilated into organic forms such as glutamine. 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). In this thesis (**Chapter 5**), I studied a key enzyme in the purine pathway that produces usable nitrogen (ammonium) through allantoate breakdown (AtAAH). Mutating this gene caused a strong reduction of several amino acids (serine, threonine, isoleucine and glycine). We hypothesize that ammonium shortage is the reason for the lower abundance of the amino acids in *Ataah* seeds and, consequently, this might act to block pathways leading to the completion of germination, for example the inhibition of the translation of transcripts that are necessary for germination.

Both storage and non-storage proteins present in the dry mature seeds and new proteins that are synthesized upon imbibition are important for the success of germination (Rajjou et al., 2004). These proteins, like other key macromolecules (i.e. nucleic acids and membrane lipids), are easy targets of oxidative stress during maturation, drying and germination. Excessive protein oxidation may prevent the normal functioning of targeted seed proteins and enzymes and/or increase their susceptibility towards proteolytic degradation and, consequently, loss of germination ability. Since protein oxidation mainly results from reactions with reactive oxygen species (ROS), this highlights the potential role of antioxidant systems through detoxification and protection to maintain seed vigour. Seeds are endowed with a large number of detoxifying enzymes that scavenge ROS during dry maturation and germination (Oracz et al., 2007). If these enzymes were to undergo some damage during seed storage, imbalance between the production of ROS and scavenger activity may lead to a reduction of seed vigour. NADP-ME1 is an enzyme that generates NADPH through decarboxylation of malate to pyruvate. Since seeds of the NADP-ME1 loss-of-function mutant showed reduced seed viability relative to the wild type (**Chapter 4**), it is hypothesized that NADPH generated by this enzyme provides reducing power for antioxidant enzymes. Furthermore, the observed increase in protein oxidation (carbonylation), at different storage times in *nadp-me1* mutant seeds, provides support to the finding

that oxidative stress accompanies higher rates of seed aging in the mutant. Since carbonylation is an unrepairable modification and carbonylated proteins are marked for proteolysis (Kalemba and Pukacka, 2014), higher levels of amino acid accumulation in aged mutant seeds at 6 hours after sowing (6HAS) is probably due to enhanced protein degradation. That this finding is most severe for the 6 HAS time-point (**Chapter 4**) is probably due to the fact that the samples analysed for this time-point consist of a mixture of viable and non-viable seeds, which is different for the later time-points were the non-germinating seeds have been removed. Likely these free amino acids could not be incorporated into newly synthesized proteins due to damage to the translational machinery, as shown for the potential of de novo protein synthesis and translational activity, which was severely reduced during both artificial and natural aging (Rajjou et al., 2008).

Concluding remarks

The biological studies that I have presented in this thesis demonstrate the utility of natural variation in combination with transcriptome analysis in discovering genetic variation underlying phenotypic differences. This approach, using dormancy natural variants and reverse genetics has led to the identification of a set of genes that contribute to seed dormancy and germination phenotypes. In this thesis we chose three genes for deeper analysis. However, further extensive experiments and other methodologies can be applied to exploring their function more precisely. In addition, the other genes that I identified also provide a solid resource for further study to elucidate their causal molecular mechanisms. Hopefully, potential candidates may be used to develop seeds with new agronomic traits and better seed performance to improve yield potential and stability.

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Summary

Fundamental knowledge about the processes affecting seed performance, including the regulation of germination, dormancy and longevity can provide insight to improve these traits, which is of economic importance for agricultural use and storage of seed crops. Accordingly, the objective of this study was to reveal genetic regulators for seed dormancy and possibly other traits related to seed performance. For this purpose the study started with the transcriptome profiling of freshly harvested (dormant) and after-ripened (AR; non-dormant) 24-hour imbibed seeds of four different *DELAY OF GERMINATION* near isogenic lines (*DOG*NILs) and the Landsberg *erecta* (L*er*) wild type, which have different levels of primary dormancy. This comparative transcriptomics approach lead to the identification of genes that were either related to seed dormancy or to after-ripening in all of these genotypes (46 and 25 genes for the dormancy-up and after-ripened-up set, respectively). The study was followed by reverse genetic analysis in the model plant *Arabidopsis thaliana* and resulted in several dormancy and other germination-related phenotypes (**Chapter 2**). Three mutants displaying the most interesting phenotypes were selected for in depth studies of their corresponding genes.

In **Chapter 3** we studied *FAST GERMINATING SEEDS* (*FGS*), a novel gene whose lack of function mutant seeds displayed reduced seed dormancy, faster and more uniform germination and lower sensitivity to abiotic stress, while seed longevity was not affected. The germination phenotypes represented by the *fgs* mutant resemble the requirements for vigorous seeds in an agricultural context and make *FGS* an interesting candidate gene for the seed industry. By genetic complementation of the *fgs* mutant we showed that the mutant phenotype is indeed due to disruption of the *FGS* gene. The expression of *FGS* mRNA is induced during seed maturation and decreases upon seed imbibition. The *fgs* mutant was further investigated using metabolomics and proteomics analysis which revealed a reduced abundance of seed storage related proteins in dormant dry seeds and a quicker release of amino acids in germinating seeds in comparison to wild type Columbia. Furthermore, reciprocal crosses indicated that FGS controls seed germination through the maternal tissue. These findings together with localization of *FGS* in protein storage vacuoles suggests a role for *FGS* in seed storage protein mobilization.

In **Chapter 4,** I studied the role of the *NADP-MALIC ENZYME 1* (*NADP-ME1*) gene in seed longevity. Loss-of-function of *NADP-ME1* resulted in a reduced seed viability relative to wild type. *NADP-ME1* is one of four Arabidopsis NADP-ME isoforms that catalyses the oxidative decarboxylation of malate to yield pyruvate, CO_2, and NADPH. The effect on seed longevity is specific for *NADP-ME1* as was revealed by mutant analyses and complementation cloning. Furthermore, dry *nadp-me1* mutant seeds display higher levels of protein carbonylation than wild type and upon seed imbibition malate and amino acids accumulate in the embryos of aged mutant seeds compared to wild type. *NADP-ME1* expression is increased in imbibed aged as compared to non-aged seeds and NADP-ME1 activity at testa rupture promotes normal germination of aged seeds. Moreover, in seedlings of aged seeds NADP-ME1 is specifically active in the root meristematic zone. We propose a role for NADP-ME1 in the protection of seed proteins against oxidative damage.

The last gene that I studied is *ALLANTOATE AMIDOHYDROLASE* (*AtAAH*) that encodes an enzyme in the uric acid catabolic pathway. Seeds carrying mutations in this gene display increased seed dormancy. Since AtAAH is a key enzyme in the pathway that produces usable nitrogen for subsequent anabolic reactions, I hypothesized that higher dormancy of the *Ataah* mutant seeds is the consequence of a defective nitrogen production. To test this hypothesis, exogenous nitrate was applied during seed maturation and seed germination to see whether it can rescue the germination behaviour of the mutant. Results showed that applying exogenous nitrate during both seed maturation and germination partially complement the higher dormancy phenotype of the mutant seeds. Based on these findings we conclude that a defective AtAAH leads to a reduction in nitrogen (ammonia) release in freshly harvested seeds. This causes a block of germination which can be overcome by seed after-ripening or by the application of exogenous nitrate (**Chapter 5**).

Finally, I integrated and discussed the work presented in this thesis in **Chapter 6**. The power of our approaches (multi-NIL transcriptomic analysis) in search for candidate genes was emphasized, as knock-out mutants in several genes showed dormancy and germination-related phenotypes. I also discussed the potential of this knowledge for improving traits that contribute to seed performance including seed dormancy, seed longevity, germination rate, pre-harvest sprouting and germination under stress condition. In addition I described the processes and strategies that can

be used for translating the knowledge gained form this thesis to improve crops species.

Samenvatting

Fundamentele kennis met betrekking tot processen die zaadeigenschappen beïnvloeden, zoals de regulatie van kiemrust en bewaarbaarheid, kan leiden tot inzichten die gebruikt kunnen worden voor het verbeteren van deze eigenschappen. Dit is van economisch belang zowel voor de landbouw als voor het bewaren van gewaszaden. Het doel van deze studie is daarom het onthullen van genen die kiemrust en andere zaadeigenschappen reguleren. Hiervoor zijn eerst de transcriptome profielen van vers geoogst (dormant) en nagerijpt (niet dormant) zaad van vier verschillende *DELAY OF GERMINATION* bijna isogene lijnen en wildtype Landsberg *erecta* (L*er*) bepaald. Deze lijnen hebben verschillende kiemrust niveaus. Vergelijkend onderzoek met deze transcriptomen heeft tot de identificatie geleid van genen die ofwel aan kiemrust of wel aan narijping gerelateerd zijn (46 genen opwaarts gereguleerd tijdens kiemrust en 25 genen opwaarts gereguleerd in nagerijpt zaad). Deze studie werd voortgezet met een "reverse genetic analysis" in de modelplant *Arabidopsis thaliana* en heeft geleid tot de identificatie van verscheidene mutanten met kiemrust en andere kieming gerelateerde fenotypen (**Hoofdstuk 2**). Drie mutanten met de meest interessante fenotypes zijn vervolgens geselecteerd voor verdere analyse.

In **Hoofdstuk 3** heb ik *FAST GERMINATING SEEDS* (*FGS*) nader onderzocht. *FGS* mutanten produceren zaden met minder kiemrust, een snellere en meer uniforme kieming en een lagere gevoeligheid voor abiotische stres, terwijl de bewaarbaarheid van deze zaden niet veranderd is. De kiemingsfenotypen van de *fgs* mutant reflecteren de vereisten die gesteld worden aan vitale zaden in de landbouw en maken *FGS* daarom een interessante kandidaat voor de zaadindustrie. Middels genetische complementatie hebben we laten zien dat het *FGS* gen inderdaad gemuteerd is in de knock-out lijn. *FGS* mRNA komt tijdens de zaadrijping tot expressie en verdwijnt weer tijdens de zaadimbibitie. Metabolome en proteome analyses op zaden van de *fgs* mutant laten zien dat de vers geoogste zaden minder opslageitwitten bevatten en dat aminozuren sneller vrijkomen tijdens de imbibitie in vergelijking met Columbia wildtype zaden. Daarnaast blijkt uit de analyse van reciproke kruisingen dat *FGS* de kieming via het maternale weefsel beïnvloedt.

Gecombineerd met de lokalisatie van FGS in eiwit opslagvacuolen suggereert dit een rol voor FGS in de mobilisatie van opslageiwitten.

In **Hoofdstuk 4** heb ik de rol die *NADP-MALIC ENZYME 1* (*NADP-ME1*) speelt in zaadbewaarbaarheid onderzocht. *NADP-ME1* mutantzaden zijn minder goed bewaarbaar in vergelijking tot wildtype zaden. *NADP-ME1* is één van de vier Arabidopsis NADP-ME isovormen die de oxidatieve decarboxylering van malaat naar pyruvaat katalyseren; hierbij komen CO_2, en NADPH vrij. Het effect op bewaarbaarheid is specifiek voor *NADP-ME1* zoals blijkt uit de mutantanalyses en de complementatie testen. Verder blijkt dat droge zaden van de *nadp-me1* mutant een hogere eiwit carboxylering hebben dan wildtype zaden en dat tijdens de imbibitie van verouderde zaden malaat en aminozuren accumuleren in mutantzaden in vergelijking met wildtype. *NADP-ME1* genexpressie is hoger in geïmbibeerde verouderde zaden in vergelijking tot niet verouderde zaden en NADP-ME1 activiteit tijdens het doorbreken van de zaadhuid door het wortelpuntje (kieming *senso stricto*) en stimuleert de kieming van verouderde zaden. NADP-ME1 activiteit in zaden is specifiek voor de meristimatische zone in de wortel. Ik vermoed dat NADP-ME1 een rol speelt bij de bescherming van eiwitten tegen oxidatieve beschadigingen in zaden.

Het laatste gen dat ik bestudeerd heb is *ALLANTOATE AMIDOHYDROLASE* (*AtAAH*), een enzym uit de urinezuur biosynthetische reactieroute. Zaden met mutaties in dit gen hebben een verhoogde kiemrust. AtAAH speelt een sleutelrol bij de productie van stikstof, vandaar dat ik denk dat de verhoogde kiemrust in de *Ataah* mutant veroorzaakt wordt door een defectieve nitraatproductie. Voor het testen van deze hypothese hebben wij gekeken of toevoeging van nitraat tijdens de zaadrijping en kieming leid tot een betere kieming. De resultaten geven aan dat toevoeging van nitraat gedeeltelijk de diepere kiemrust van de mutant zaden kan overkomen. Gebaseerd op deze vindingen concluderen wij dat een defect AtAAH leidt tot een reductie van stikstok (ammonia) in net geoogste rijpe zaden, en daardoor een diepere kiemrust. Deze blokkade kan dus worden verholpen door de zaden te laten narijpen of door nitraat toe te voegen (**Hoofdstuk 5**).

Tot slot heb ik het werk dat in dit proefschrift gepresenteerd is geïntegreerd en bediscussieerd in **Hoofdstuk 6**. De efficiëntie van de methoden (multi-NIL transcriptome analyses) die voor dit onderzoek gebruikt zijn voor het identificeren van kandidaat genen blijkt uit het grote aantal geïdentificeerde mutanten met kiemrust en andere kiemings gerelateerde fenotypes. Ik beschrijf ook de potentie van deze kennis voor het verbeteren van eigenschappen zoals kiemrust, bewaarbaarheid, kiemsnelheid, onrijpe kieming en kieming in stress condities. Daarnaast beschrijf ik de processen en strategieën die gebruikt kunnen worden om deze kennis te gebruiken voor het verbeteren van landbouwgewassen.

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After a prolonged period of almost seven years, today is the day to offer my sincere thanks to all who have supported and helped me scientifically and emotionally throughout my PhD studies. First, my deepest gratitude to God for His blessings throughout my life and ever more in this study.

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During my journey towards this degree, I had the pleasure of working with a teacher, a friend, an inspiration, a role model and a pillar of support in my Guide, Dr. Leónie Bentsink. It is indeed my proud privilege to express my deep sense of gratitude to her. She provided her genuine support and supervision at all time of research and writing of this thesis that guided me to grow as a research scientist. I appreciate all her contributions of time, ideas, and funding to make my Ph.D. experience productive and exciting. Dear Leónie, I appreciate your great knowledge and your serious attitude towards your work, but at the same time your kindness and care. To give an example, I remember the day of our lab trip that during our talk I asked you about Dutch translation of "starch" which I needed to buy for cooking, and the day after that when I came to the office I saw several box of starch on my desk and a note from you that really surprized me. I have learned a lot from you. Thank you for every things!

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that one day you can read this book and understand why I spent so much time in front of my computer.

Last but not least, I would like to express my sincere thanks to my beloved husband, for being so understanding and patient and for his faithful support during all stages of my research. My heartfelt thanks for everything!

Curriculum Vitae

Farzaneh Yazdanpanah was born on the 11th of August 1978 in Abadan, Iran. In 1997 she successfully passed the national exam for universities and started her 4-years bachelor study on agronomy and plant breeding in Shiraz University. After her graduation, she passed the national exam in 2003 and started her MSc study at the major of Agricultural Biotechnology in the College of Agriculture, Hamadan University. She performed her MSc project in the Agricultural Biotechnology Research Institute of Iran (ABRII) on the project of ''Enhanced insect resistance to bollworm (*Helicoverpa armigera*) in cotton containing a synthetic *cry1Ab* gene'' under the supervision of Dr. Masoud Tohidfar. After her MSc degree in 2006, because of her passion to philosophy, she started a BSc study in this field. In 2010, she moved to Netherlands with her husband who was awarded a scholarship to pursue his PhD at Wageningen University. In April 2011, Farzaneh started working as a PhD student in Wageningen Seed Lab, Laboratory of Plant Physiology on the project entiteled ''Molecular and phenotypical characterization of genes involved in the control of seed dormancy and after-ripening'' under the supervision of Dr. Leónie Bentsink and Dr. Henk Hilhorst. The results of this project are described in this thesis.

List of publications

1. **Yazdanpanah F**, Hanson J, Hilhorst HW, Bentsink L (2017). Differentially expressed genes during the imbibition of dormant and after-ripened seeds–a reverse genetics approach. BMC Plant Biology 17: 151

2. Costa MCD, Righetti K, Nijveen H, **Yazdanpanah F**, Ligterink W, Buitink J, Hilhorst HW (2015). A gene co-expression network predicts functional genes controlling the re-establishment of desiccation tolerance in germinated Arabidopsis thaliana seeds. Planta 242: 435-449

3. **Yazdanpanah F**, Tohidfar M, Esna AM, Ghareyazi B, Karimi Jashni M, Mosavi M (2009). Enhanced insect resistance to bollworm (*Helicoverpa armigera*) in cotton containing a synthetic *cry1A(b*) gene. Indian Journal of Biotechnology,8:72–77

Education Statement of the Graduate School

Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to:	**Farzaneh Yazdanpanah**
Date:	**20 March 2018**
Group:	**Plant Physiology**
University:	**Wageningen University & Research**

1) Start-up phase	*date*
▶ **First presentation of your project**	
Title: Molecular and phenotypical characterization of genes that are involved in the control of	17 Oct 2011
▶ **Writing or rewriting a project proposal**	
Title: Molecular and phenotypical characterization of genes that are involved in the control of	25 Jul 2011
▶ **Writing a review or book chapter**	
▶ **MSc courses**	
▶ **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7.5 credits**

2) Scientific Exposure	*date*
▶ **EPS PhD student days**	
EPS PhD student day, Wageningen, NL	20 May 2011
EPS PhD student day, Amsterdam, NL	30 Nov 2012
▶ **EPS theme symposia**	
EPS theme 2 'Interactions between Plants and Biotic Agents', Wageningen, NL	10 Feb 2012
EPS theme 3 'Metabolism and Adaptation', Utrecht, NL	26 Apr 2012
EPS theme 1 'Developmental Biology of Plants', Wageningen, NL	24 Jan 2014
EPS theme 3 'Metabolism and Adaptation', Wageningen, NL	10 Feb 2015
▶ **National meetings (e.g. Lunteren days) and other national platforms**	
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	04-05 Apr 2011
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	02-03 Apr 2012
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	22-23 Apr 2013
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	13-14 Apr 2015
Seminars (series), workshops and symposia (highly recommended)	
seminar of invited speaker (Steven Penfield)	12 Jun 2012
seminar of invited speaker (Aaron Fait)	26 Jun 2012
seminar of invited speaker (Andrew Sugden)	04 Dec 2012
seminar of invited speaker (Prof. Marc Boutry)	08 Feb 2013
workshop of 'Reviewing a Scientific Paper'	08 Nov 2012
Dies Natalis Lustrum symposium	15 Mar 2013
2nd Dutch Seed Symposium, Wageningen, NL	01 Oct 2013
4th Dutch Seed Symposium, Wageningen, NL	06 Oct 2015
▶ **International symposia and congresses**	
23rd International Conference on Arabidopsis, Vienna, Austria	03-07 Jul 2012
4th Workshop on the Molecular Aspectsof Seed Dormancy and Germination, Paris, France	09-12 Jul 2013
▶ **Presentations**	
Poster: Annual meeting 'Experimental Plant Sciences', Lunteren, NL	02-03 Apr 2012
France	09-12 Jul 2013
Poster: Annual meeting 'Experimental Plant Sciences', Lunteren, NL	13-14 Apr 2015
Talk: EPS Theme 3 symposium, Wageningen, NL	10 Feb 2015
Talk: PhD trip, Syngenta, Stein, Switzerland	30 Apr 2015
▶ **IAB interview**	
Meeting with a member of the international Advisory Board of EPS	05 Jan 2015
▶ **Excursions**	
PPO-locations, Vredepeel, NL	15 Jul 2011
Excursion day to Rijk Zwaan, De Lier, NL	27 Sep 2013
PhD trip visiting scientific institutes, universities, companies in NL, Germany and Switzerland	22 Apr-01 May, 2015
Subtotal Scientific Exposure	*15.8 credits**

3) In-Depth Studies		*date*
▸	**EPS courses or other PhD courses**	
	Master Class Seed Technology, Wageningen, NL	12-16 Oct 2014
	PhD course: 'Bioinformatics - a user's approach', Wageningen, NL	26-30 Aug 2013
▸	**Journal club**	
	Literature discussions in Plant Physiology	2011-2015
▸	**Individual research training**	
	Subtotal In-Depth Studies	*6.0 credits**
4) Personal development		*date*
▸	**Skill training courses**	
	English Speaking and Listening III	Sep 2011-Feb 2012
	Workshop Presentation Skills	Mar-Apr 2012
	English course for IELTS, Radboud University, Nijmegen	Mar-Jun 2013
	Techniques for Writing and Presenting a Scientific Paper	Apr 16-19, 2013
▸	**Organisation of PhD students day, course or conference**	
▸	**Membership of Board, Committee or PhD council**	
	Subtotal Personal Development	*7.1 credits**
	TOTAL NUMBER OF CREDIT POINTS*	**36.4**
Herewith the Graduate School declares that the PhD candidate has complied with the educational		
** A credit represents a normative study load of 28 hours of study.*		

The research was conducted at the Laboratory of Plant Physiology of Wageningen University and financially supported by Wageningen University.

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Propositions

1. Among the four Arabidopsis *NADP-MALIC ENZYME* (*AtNADP-MEs*) genes, only *AtNADP-ME1* affects germination after seed storage.
   (this thesis)
2. Proteome and metabolite analysis of the *FAST GERMINATING SEEDS* (*FGS*) loss-of-function mutant, together with localization analysis for the encoded protein, suggest that FGS has a role in seed storage protein mobilization.
   (this thesis)
3. Increased expression of genes during a specific plant developmental stage does not necessarily mean that those genes are required for that stage.
4. The current situation of farming in many countries necessitates more emphasis on applied research and agricultural education.
5. Access to factual news should be a basic human right, but this is often neglected by media.
6. A positive attitude by the teacher affects students' performance and leaves them with memories that follow them during their lives.

Propositions belonging to the thesis, entitled

'Identification and functional analyses of genes regulating seed dormancy, longevity and germination'.

Farzaneh Yazdanpanah

Wageningen, 20 March 2018.