Adaptation and acclimation of seed performance

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Chapter 1

General Introduction
Chapter 1

The model plant Arabidopsis

Arabidopsis thaliana (Arabidopsis) is a member of the Brassicaceae family with a vast natural distribution through Europe, Asia and North America (Meinke et al., 1998). It is genome was the first plant and the third multicellular organism after Caenorhabditis elegans and Drosophila melanogaster to be completely sequenced (Kaul et al., 2000). Arabidopsis is a small plant with short life cycle and possesses a fully sequenced small genome (125Mbp) made up of only five chromosomes and more than 27,000 protein coding genes (Kaul et al., 2000; Haberer et al., 2011). The accumulation of knowledge, biological resources and available molecular tools add up to the attractiveness of Arabidopsis as a model system (Alonso & Ecker, 2006). Moreover, knowledge of Arabidopsis can be transferred to important crops which are of commercial interest, such as tomato, pepper, Brassica, cucumber, maize, rice, soybean, barley, rye, wheat and many other crops (Zhang & Blumwald, 2001; Chew & Halliday, 2011; Piquerez et al., 2014).

Natural variation in Arabidopsis

Arabidopsis is an annual species which can be found in the northern hemisphere and occupies contrasting habitats along latitude, longitude and altitude. This geographic variation influences the life cycle which is an adaptation feature that allows plants to survive in harsh environments and to propagate when the conditions are suitable for growth (Chiang et al., 2011). Arabidopsis accessions display winter or spring annual life cycles (Donohue, 2009). Seeds from accessions with a winter life cycle germinate in autumn. During the winter, seedlings or rosettes grow slowly, and only in spring, when temperatures start to get warmer, the plants flower and disperse new seeds (offspring). For accessions with a spring life cycle the seeds germinate in spring and the seedlings grow into mature plants and disperse their offspring in the same spring or summer. These new seeds overwinter and do not germinate, remaining dormant until the following spring (Donohue, 2009).

Arabidopsis accessions show variation for many (economically important) traits, such as plant resistance to drought, seed yield and seed quality (Verslues & Juenger, 2011; Chardon et al., 2012; Assmann, 2013). Knowledge of the genes affecting these traits is very useful since genetic variation can then be used in breeding programs to improve cultivars, especially for food and seed production. Natural genetic variation can be examined by Quantitative Trait Locus (QTL) analysis, which is a tool frequently used in Arabidopsis over the past decades (Alonso-Blanco & Koornneef, 2000; Paran & Zamir, 2003; Alonso-Blanco et al., 2009; Weigel, 2012). Populations of recombinant inbred lines (RILs) have been used most frequently in Arabidopsis because of the advantages derived from their homozygosity (Bentsink & Koornneef, 2011). RILs are obtained by single-seed descent from F2 plants until the F9 or further generations. These populations are highly suitable to
map QTLs because the influence of the environment on the quantitative trait can be reduced by assessing multiple individuals of the same genotype instead of just a single plant (Bentsink & Koornneef, 2011). For confirmation of the presence and the effect of a QTL, near isogenic lines (NILs) are used. NILs contain an introgression of one parent’s alleles at a QTL position into the genetic background of the other (recurrent) parent (Reymond et al., 2007). NILs can be used for the identification of the genes underlying the QTL by map based cloning (Alonso-Blanco & Koornneef, 2000), but also for physiological analysis of the phenotypes. For example, Alonso-Blanco et al. (2003) identified seven dormancy QTLs called Delay of Germination (DOG). Using different NILs carrying specific Cape Verde Islands (Cvi) introgression fragments in a Landsberg erecta (Ler) genetic background that showed different dormancy behaviour, they could confirm four of the QTLs. In addition, Bentsink et al. (2010) confirmed nine DOG QTLs using a set of NILs constructed by the introgression of the DOG loci of different accessions into a Ler genetic background.

Besides experimental populations, also natural accessions can be used to identify loci controlling traits of interest, using genome-wide association (GWA) mapping. GWA mapping has shown to be a robust tool to study intraspecific genetic variation that controls phenotypic variation of plant populations (Bergelson & Roux, 2010; Atwell et al., 2010). In GWA the link between genotypes at the markers and phenotypes of the desired trait can be identified using statistical tests (Alonso-Blanco & Koornneef, 2000; Bac-Molenaar et al., 2015). For Arabidopsis there are different types of natural populations available, including worldwide collections such as the so-called Hapmap population, for which over 750 natural accessions have been collected from around the world (Weigel & Mott, 2009), and more structured populations of Arabidopsis accessions, such as the population collected in France (Le Corre, 2005; Brachi et al., 2013) and in the Iberian Peninsula (Picó et al., 2008; Méndez-Vigo et al., 2011; Manzano-Piedras et al., 2014). The use of structured populations gives better insight in climatic clines (Méndez-Vigo et al., 2011; Manzano-Piedras et al., 2014). Studies using more structured populations showed more consistent patterns (Kuittinen et al., 2002; Le Corre, 2005; Méndez-Vigo et al., 2012), whereas studies using worldwide accessions showed greater gene-to-gene variation in their level and pattern of variation (Shepard & Purugganan, 2003).

**Seed performance**

**What is seed performance?**

Seeds are important for humans across the world, since they can be a source of medicine, oil, fibre (e.g. cotton), feed and food. A 60% increase in food production must be attained by 2050 when global population growth will have resulted in over 9 billion humans inhabiting the world (FAO, 2015). Plants account for more than 80% of the human diet, and seeds account for 60% of the energy intake of the world population, mainly by five cereals
(rice, wheat, maize, millet and sorghum) (FAO, 2015). Next to that, seeds are an important commodity and for this, seeds with high performance are vital. Seed performance is determined by a combination of factors including: genetic homogeneity, physical appearance, viability, vigor, uniformity, dormancy and longevity (Basra, 2006).

Two main factors affecting seed performance are longevity and dormancy. By definition, seed longevity is the capacity of the seed to germinate after long-term storage and is measured by the rate of deterioration, aging and loss of vigor during or after storage (Bernal-Lugo & Leopold, 1992; Bewley et al., 2013). Seed dormancy prevents germination even though conditions are suitable (relating to water, light, temperature and gaseous conditions) (Fenner & Thompson, 2006). Seed dormancy contributes to the adaptation of plants to their environment by optimizing the germination to the right period of the year (Donohue et al., 2005). The level of dormancy in seeds is determined by several factors such as genetic origin and the environmental factors operating during development and maturation (Gutterman, 2000; Fenner & Thompson, 2006; Bewley et al., 2013).

Seed dormancy can be released by dry after-ripening (AR) during several months of mild/warm temperatures or by stratification during low-temperature treatment (cold stratification) of imbibed seeds (Bewley, 1997; Bewley et al., 2013). Cold stratification may induce biosynthesis of gibberellins (GAs) which, in turn, promotes seed germination (Yamauchi et al., 2004). The molecular mechanisms of dry after-ripening are not understood yet (Finch-Savage et al., 2007), but it is known that abscisic acid (ABA) content decreases (Tillberg & Pinfield, 1982; Ali-Rachedi et al., 2004; Gubler et al., 2005; Millar et al., 2006). The phytohormone ABA is derived from carotenoids and is a key hormone in seed dormancy (Kendall et al., 2011). ABA induces dormancy during seed maturation and also maintains the dormant state in imbibed seeds and is hence an antagonist of GA (Hilhorst, 1995; Bewley, 1997; Holdsworth et al., 2008). Analysis of mutants and transgenic plants of Arabidopsis has provided strong evidence that ABA biosynthesis and responses to this phytohormone are involved in the onset and maintenance of dormancy (Gubler et al., 2005). Endogenous ABA levels can change strongly during seed development, germination, and post-germination growth in response to developmental and environmental cues (Okamoto et al., 2006; Bentsink & Koornneef, 2008).

Recent studies have demonstrated that dormancy and longevity correlate negatively; seeds with deep dormancy exhibit low longevity and seeds with shallow dormancy display better storability (Nguyen et al., 2012; He et al., 2014). This correlation was corroborated by the colocation of the QTLs GAAS (Germination Ability After Storage) and DOG in six RIL populations and confirmed by the analyses of a set of NILs. Moreover, it was shown that one of the QTLs, DOG1, affected seed dormancy and longevity (Nguyen et al., 2012).
The effect of the environment on seed performance

Over the last decades climate change has been affecting agricultural productivity, reducing yield in many cases (Gornall et al., 2010). Phenological traits, such as morphology and physiology are very sensitive to changes in temperature, water, photoperiod and nutrients, and therefore may lead to problems in the reproduction of the species and, concomitantly, reduced seed performance (Field & Lake, 2011; Singh et al., 2013). In depth studies are required to fully understand the complex interactions between environmental and genetic factors that regulate seed performance.

The role of temperature, light quality, day length, water and nutrients in determining the degree of dormancy has been investigated in a wide range of species (Fenner, 1991; Hilhorst, 1995; Baskin & Baskin, 2014). Temperature during seed development and maturation is one of the most important determinants of seed dormancy in many species (Fenner, 1991; Biddulph et al., 2007; Llorens et al., 2008; Javaid et al., 2010; Kendall & Penfield, 2012; Huang et al., 2014; He et al., 2014). In a variety of species, including Beta vulgaris, Lactuca sativa, Amaranthus retroflexus, wild oat, Avena fatua (Fenner, 1991) and Arabidopsis (Kendall et al., 2011; Penfield & Springthorpe, 2012; Huang et al., 2014; He et al., 2014), warmer temperatures during seed development generally produce seeds that are less dormant than those that were developed at cooler temperatures.

The performance of the progeny can also be affected by day length during the final stages of seed maturation (Gutterman, 2000). Germinability of some species has been reported to be higher for seeds produced under shorter days (Gutterman, 1973; Gutterman, 1978; Munir et al., 2001); however Contreras et al. (2008) and He et al. (2014) did not find any significant effect of day length on seed performance.

Another important key factor for vigorous germination and successful seedling establishment is the nutrition of the mother plant during seed formation. The accumulation of nutrients in seeds is a complex process and depends on both environmental and genetic factors (Papdi et al., 2009). The supply of fertilizers to parental plants changes the seed dormancy of several species (Fenner, 1991). Low potassium availability during seed development of Sorghum bicolor increased germinability which correlated with reduced ABA content of the seeds (Arnold et al., 1995). In Arabidopsis, nitrate treatment of the mother plant led to metabolic changes in the seeds, enabling the seeds to overcome the inhibition imposed by ABA by enhancing its degradation (Matakiadis et al., 2009). The depth of seed dormancy is inversely correlated with the nitrate dose applied to the mother plant during seed formation. Low nitrate, thus, resulted in the production of more dormant seeds in Arabidopsis (Alboresi et al., 2005; He et al., 2014) while high nitrate application to Sisymbrium officinale mother plants resulted in less dormant seeds (Bouwmeester et al., 1994). Molybdenum deficiency in wheat also resulted in low seed dormancy by decreasing the abscisic acid content (Modi & Cairns, 1994). It is likely that a deficiency of
molybdenum, a co-factor of nitrate reductase (Mendel & Hänsch, 2002), reduces nitrate reductase activity, resulting in higher nitrate levels in seeds and, hence, lower dormancy. Phosphate levels did not influence seed dormancy in Arabidopsis, but higher seed phosphate and phytate content increased germination ability under stress conditions (He et al., 2014).

**Loci affecting seed performance**

Many studies in Arabidopsis have contributed to the understanding of the processes affecting seed performance, including the regulation of germination, dormancy, longevity and the acquisition of desiccation tolerance (Baud et al., 2002; Bentsink & Koornneef, 2008; Maia et al., 2011; Nguyen et al., 2012; Dekkers et al., 2013; He et al., 2014; Dekkers & Bentsink, 2015). Using a RIL population of Landsberg erecta (Ler) and Cape Verde Islands (Cvi), Alonso-Blanco et al. (2003) identified the main dormancy QTL described so far, DOG1, that was cloned by map-based cloning and encodes a protein of unknown function (Bentsink et al., 2006). The dog1 mutant shows absence of dormancy after harvest, indicating that DOG1 is a key player in the induction of seed dormancy. The level of DOG1 protein is highly correlated with the depth of dormancy at the end of seed maturation (Nakabayashi et al., 2012). In addition to DOG1, ten other DOG QTLs affecting seed dormancy were identified by Bentsink et al. (2010). Nine of these QTLs have been confirmed by NILs carrying introgression fragments of different accessions in a Ler genetic background. NIL DOG1, 3 and 6 showed an increase in seed dormancy in comparison with Ler, whereas in NIL DOG2 and 22 the dormancy level was decreased (Bentsink et al., 2010; He et al., 2014).

In addition, mutant approaches and/or overexpression of the genes of interest have identified genes with large effects on phenotype (or trait value) (Reymond et al., 2007). Knock-out mutants are extremely useful, because they provide a direct route to determine the function of a gene in situ (Krysan et al., 1999) and generate a phenotype distinct from the wild type; however, in most cases, the effect of the studied gene is not easily detectable at the morphological level, and a specific test is required to detect the difference between the mutant and the wild type, e.g. a germination assay to detect dormancy phenotypes (Reymond et al., 2007). By using T-DNA knock-out- or overexpressor mutants, several genes that can affect seed performance have been identified, e.g. mutants of genes regulating seed maturation, such as leafy cotyledon 1 and 2 (lec1, lec2), fusca 3 (fus3), abscisic acid insensitive 3 (abi3) and the mutants of the Late Embryogenesis Abundant (LEA) family (Raz et al., 2001; Hundertmark & Hincha, 2008; van Zanten et al., 2011); mutants that affect seed germination or seed dormancy, such as histone monoubiquitination 1 (hub1), abscisic acid insensitive 4 and 5 (abi4, abi5) and reduced dormancy 2 (rdo2) (Finkelstein, 1994; Finkelstein & Lynch, 2000; Peeters et al., 2002; Liu et al., 2007) and mutants that affect seed longevity, such as frostbite 1 (fro1), transparent testa (tt), aberrant
testa shape (ats) and phospholipase D alpha 1 (pldα1) (Debeaujon et al., 2000; Clerkx et al., 2004; Devaiah et al., 2007). Because the number of genes that affect seed performance is high, I will here focus on the genes that are studied in this thesis.

**Genes involved in ABA biosynthesis and catabolism**

Molecular genetic analyses has indicated that 9-cis-epoxycarotenoid dioxygenases (NCEDs) catalyze the first step of ABA biosynthesis and, hence, NCED genes may be key elements in the control of ABA content in seeds (Tan et al., 2003; Lefebvre et al., 2006). The NCED genes regulate physiological processes such as seed development, maturation, desiccation and germination by affecting the ABA concentration in seeds (Tan et al., 2003; Lefebvre et al., 2006). ABA levels are reduced in the seeds of the Atnced6 and Atnced9 mutants and these mutant seeds are resistant to paclobutrazol, an inhibitor of gibberellin biosynthesis, that normally inhibits germination (Lefebvre et al., 2006). Although seeds of the single mutants were still dormant, reduced dormancy was clearly observed in the Atnced6 Atnced9 double-mutant seeds.

The CYP707A genes belongs to the large group of Cytochrome P450s and encode enzymes of the ABA catabolic pathway, thus playing a prominent role in regulating endogenous ABA levels during seed development and germination (Okamoto et al., 2006). Transcript abundance of CYP707A genes increases in response to abiotic stress, dehydration and exogenous ABA treatment (Saito et al., 2004). T-DNA insertion mutants of CYP707A2 in Arabidopsis have higher ABA content in seeds and exhibit increased dormancy as compared to the wild type (Kushiro et al., 2004). CYP707A1 is expressed predominantly during mid-maturation and is down-regulated during late-maturation whereas CYP707A2 transcript levels increase from late-maturation to the mature dry seed stage, indicating that CYP707A2 plays a major role in reducing ABA content in after-ripened Arabidopsis seeds or during early seed imbibition (Kushiro et al., 2004; Okamoto et al., 2006; Matakiadis et al., 2009). The use of various genotypes (i.e. mutants of ABA biosynthesis and catabolism genes) allows us to determine which pathways are affected by the maternal environment.

**Metabolites associated with seed performance**

The acquisition of desiccation tolerance and seed dormancy in Arabidopsis is associated with an increase in most amino acids, sugars (including sucrose and raffinose), fumarate, succinate, as well as myo-inositol, sorbitol/galactitol and fatty acids during seed maturation (Baud et al., 2002; Fait et al., 2006). Howell et al. (2009) observed an increase in hexose phosphates, tricarboxylic acid cycle (TCA) intermediates and \( \gamma \)-aminobutyric acid within only one hour from the start of imbibition of rice seeds. Principal component analysis of the metabolic profiles of germinating Arabidopsis seeds, showed a clear
separation in the metabolic profile of 6-hour imbibed seeds and seeds at radical protrusion, whereas no obvious differences were detected between dry dormant and after-ripened seeds (Joosen et al., 2013).

The accumulation of the raffinose family oligosaccharides (RFOs) from middle to late stages of maturation varies from species to species (Handley et al., 1983; Kuo et al., 1988; Baud et al., 2002; Lahuta et al., 2005; Obendorf et al., 2009). The RFOs, i.e. raffinose, stachyose and verbascose, are synthesized by a set of galactosyltransferases (Peterbauer et al., 2001) and the two essential metabolites of the RFO pathway are galactinol and myo-inositol (ElSayed et al., 2014). Galactinol synthase is the first enzyme involved in the conversion of UDP-galactose to galactinol and may play a role in the regulation of the pathway (Castillo et al., 1990; Nishizawa et al., 2008; ElSayed et al., 2014). The conversion of galactinol and sucrose to raffinose and myo-inositol is catalysed by raffinose synthase. The addition of an additional galactinol to raffinose is catalysed by stachyose synthase and results in the formation of stachyose and myo-inositol, and, adding another galactinol, stachyose is converted to verbascose and myo-inositol, which is catalysed by verbascose synthase (Castillo et al., 1990; Peterbauer et al., 2001; Nishizawa et al., 2008; ElSayed et al., 2014).

High amounts of sucrose and oligosaccharides were detected in different species during the acquisition of desiccation tolerance (Kuo et al., 1988; Koster & Leopold, 1988). Slow drying of immature soybean seeds, which induced desiccation tolerance, was found to increase raffinose and stachyose, and decrease sucrose contents, whereas seeds kept at high relative humidity did not accumulate raffinose and stachyose, except for galactinol, which was the only saccharide that accumulated in seeds maintained at high relative humidity (Blackman et al., 1992). With the progress of imbibition, oligosaccharide contents decrease, which coincides with the loss of desiccation tolerance (Koster & Leopold, 1988). A decline in vigor seems to be associated with a significant decline in monosaccharide and raffinose contents during the accelerated aging of maize seeds (Bernal-Lugo & Leopold, 1992). During 12 years of storage of beech seeds, sucrose content increased and stachyose levels decreased but this did not occur until 7 years of storage: raffinose content did not change (Pukacka et al., 2009). The storability of maize seeds correlated positively with the magnitude of the glassy state and with raffinose content, but the ratio of sucrose:raffinose appeared to be a good indicator of seed storability (Bernal-Lugo & Leopold, 1995; Bernal-Lugo & Leopold, 1998). Although Buitink et al. (2000) showed that oligosaccharides do not affect the stability of the glassy state in seeds of Capsicum annum and Impatiens walleriana, the oligosaccharides disappeared and the longevity decreased upon priming. Thus, so far, there is no definite proof that the accumulation of sugars plays a significant role in seed longevity.
Scope of the thesis

Climate plays a powerful and diverse role in ecosystems all over the world. These changes, including the change of temperature, water availability, light and nutrients during seed development, maturation and after dispersal, can strongly affect seed performance. The naturally occurring genetic variation for seed performance presumes that this trait is involved in the adaptation to different climatic factors. The environmental factors that might shape this genetic variation, as well as the molecular basis of climatic adaptation are still unknown.

In my research I used three different strategies to investigate the influence of environmental conditions on seed performance in Arabidopsis. For the first strategy, seeds of a set of different genotypes were produced in different growing conditions (light intensity, photoperiod, temperature, nitrate, phosphate). At the start of flowering, plants were shifted to the different environments and plant and seed performance were studied (Chapter 2). The second strategy is shown in Chapter 3 in which I have subjected all the non-transgenic genotypes used in Chapter 2 to a field experiment. Seeds were germinated and grown into plants in different seasons of the year (summer, autumn, winter and spring) to examine the performance of the generated seeds. The third strategy is described in Chapter 4, in which I studied seed dormancy, rate of germination, seed size and flowering time, using a regional collection of 300 natural accessions of Arabidopsis from the Iberian Peninsula, a region with different climates and ecological habitats. Correlation analysis using the geographical and environmental data of these accessions with phenotypic traits was inspected to provide insight in the effect that the environment may have had on the evolution of the regulation of the different phenotypic traits.

To expand the analysis on seed performance, I raised the issue if primary metabolites of dry seeds can be associated with seed performance. A strong and positive correlation was detected between galactinol content and seed longevity (Chapter 5). This correlation was validated in two crop species: cabbage and tomato. In Chapter 6, the results of this thesis are discussed in a broader context.
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Chapter 2

Interaction between parental environment and genotype affects plant and seed performance in Arabidopsis

Abstract

Seed performance after dispersal is highly dependent on parental environmental cues, especially during seed formation and maturation. Here we examine which environmental factors are the most dominant in this respect and whether their effects are dependent on the genotypes under investigation. We studied the influence of light intensity, photoperiod, temperature, nitrate and phosphate during seed development on 5 plant attributes and 13 seed attributes, using 12 Arabidopsis genotypes that have been reported to be affected in seed traits. As expected, the various environments during seed development resulted in changed plant and/or seed performances. Comparative analysis clearly indicated that, overall, temperature plays the most dominant role in both plant and seed performance, whereas light has prominent impact on plant traits. In comparison to temperature and light, nitrate mildly affected some of the plant and seed traits while phosphate had even less influence on those traits. Moreover, clear genotype-by-environment interactions were identified. This was shown by the fact that individual genotypes responded differentially to the environmental conditions. Low temperature significantly increased seed dormancy and decreased seed longevity of NILDOG1 and cyp707a1-1, whereas low light intensity increased seed dormancy and decreased seed longevity of NILDOG3 and NILDOG6. This also indicates that different genetic and molecular pathways are involved in the plant and seed responses. By identifying environmental conditions that affect the dormancy vs longevity correlation in the same way as the earlier identified naturally occurring loci, we have here identified selective forces that probably shaped evolution for these important seed traits.

He H. *; Vidigal D.S. *; Snoek L.B.; Schnabel S.; Nijveen H.; Hilhorst H.W.M.; Bentsink L. (2014). Journal of Experimental Botany, 65, 6603-6615. (*These authors contributed equally to this work)
Chapter 2

Introduction

Seed performance refers to the capacity of seeds to germinate under various environmental conditions and represents a critical component of the plant life cycle that is of eminent ecological and agronomic importance. It has been observed that a change of temperature, photoperiod, nutrient or drought stress, during seed development, maturation and after dispersal, may strongly affect seed performance (Donohue, 2009). The principles of plasticity or adaptation of species with respect to seed performance in response to environmental changes is still largely unclear (Donohue et al., 2005b; Donohue, 2009; Walck et al., 2011). However, it is imperative to increase our knowledge as climate changes are expected to play a powerful and diverse role in ecosystems all over the world.

One of the characteristics that determines seed performance is seed dormancy. In natural environments dormancy of dry seeds can be released by storage of the seeds for several months at mild temperatures (after-ripening) or by cold stratification, which is a low-temperature treatment of imbibed seeds (Bewley et al., 2013). The roles of temperature, light quality, photoperiod, water and nutrients in determining the degree of seed dormancy have been investigated in a wide range of species (Fenner, 1991; Hilhorst, 1995; Holdsworth et al., 2008; Bewley et al., 2013). Seeds that develop at warmer temperatures are generally less dormant at maturity than those that develop at cooler temperatures, as described for Beta vulgaris, Lactuca sativa, Amaranthus retroflexus, wild oat Avena fatua (Fenner, 1991), wheat (Biddulph et al., 2007), lettuce (Contreras et al., 2009), weedy rice (Gu et al., 2006) and Arabidopsis (Donohue et al., 2008; Kendall et al., 2011; Kendall & Penfield, 2012; Huang et al., 2014). Low temperature increases abscisic acid (ABA) content during seed development in Arabidopsis; plants grown at 15°C had two-fold higher ABA content as compared to those grown at 22°C, whereas gibberellic acid (GA) -levels were reduced around 3-fold (Kendall et al., 2011). Nutrition can also affect seed dormancy; Alboresi et al. (2005) showed that the depth of seed dormancy of Arabidopsis is inversely correlated with seed nitrate content. Higher nitrate concentrations (50 mM) administered to the mother plant led to less dormant seeds than seeds produced under standard nitrate conditions (10 mM). Nitrate probably affects seed dormancy by its effect on ABA synthesis and degradation, since Matakadiadis et al. (2009) showed that increased endogenous nitrate led to lower ABA levels in Arabidopsis seeds.

In addition to an effect on seed dormancy, environmental cues during seed development can also affect other traits that contribute to seed performance, such as seed weight, seed yield, ability to germinate and longevity (or ‘storability’). A recent study has shown that some of these traits are directly linked. Prevailing stress conditions, such as high salt, osmotic stress, high and low temperature, ABA treatment and artificial aging have a negative effect on germination, whereas seed size has a negative correlation with germination in the presence of ABA but a positive correlation with the rate of germination (Joosen et al., 2012). Nguyen et al. (2012) demonstrated a negative correlation between
Parental effects on plants and seeds

seed dormancy and seed longevity (deeper seed dormancy correlated with shorter longevity and better longevity correlated with lower seed dormancy) for natural alleles of several DELAY OF GERMINATION (DOG) loci.

ABA is a major player in plant responses to various environmental stresses and this plant hormone is also thought to play a role in seed performance after environmental stress. ABA levels increase during seed maturation and in response to different abiotic stresses (Xiong, 2003), including drought, high salinity or low temperature (Iuchi et al., 2001; Kendall et al., 2011). The family of the 9-cis-epoxycarotenoid dioxygenases (NCEDs) catalyse the first committed step in ABA biosynthesis and NCED genes are key elements in the control of ABA levels in seeds (Tan et al., 2003; Lefebvre et al., 2006). The NCED genes are involved in regulating key physiological processes in seeds, such as development, maturation, desiccation and germination, by affecting the ABA concentration (Iuchi et al., 2001; Tan et al., 2003; Lefebvre et al., 2006). In Arabidopsis, NCED3 expression is induced by drought stress and the endogenous ABA content under drought stress is increased, thereby increasing seed dormancy (Iuchi et al., 2001; Frey et al., 2012). NCED6 and NCED9 have been shown to be essential for ABA production in the embryo and endosperm that imposes dormancy, whereas NCED5 fine-tunes ABA accumulation and affects seed dormancy and drought tolerance together with other NCED family members (Frey et al., 2012). Members of the CYP707A (Cytochrome P450, Family 707, Subfamily A) gene family, which catalyse steps of the ABA catabolic pathway, also play a prominent role in regulating endogenous ABA levels during seed development and germination (Okamoto et al., 2006). CYP707A transcript levels increased in response to abiotic stress, dehydration and exogenous ABA treatment (Saito et al., 2004). CYP707A1 is expressed predominantly during mid-maturation and is downregulated during late-maturation, whereas CYP707A2 transcript levels increase from late-maturation to mature dry seed, indicating that CYP707A2 plays a major role in reducing the ABA content in after-ripening Arabidopsis seeds or during early seed imbibition (Kushiro et al., 2004; Okamoto et al., 2006; Matakiadis et al., 2009). Seeds of T-DNA insertion mutants of CYP707A2 have higher ABA content and exhibit increased dormancy, as compared to wild type plants (Kushiro et al., 2004).

Here, we investigated which parental environment most strongly affected seed and plant performance and whether there are genotype by environment interactions. We used different genotypes: a set of DOG near isogenic lines (DOG-NILs; Bentsink et al., 2010)) that are known to be affected in both dormancy and seed longevity (Nguyen et al., 2012) levels by different genetic and molecular pathways, and several mutants that are defective in DOG1 gene expression (Bentsink et al., 2006) or in ABA biosynthesis (NCED6 and NCED9; (Lefebvre et al., 2006)) and catabolism genes (CYP707A1 and CYP707A2; (Saito et al., 2004; Kushiro et al., 2004)). These different genotypes might give a first indication of the genetic and molecular pathways that are involved in the response to the parental environment. A noticeable difference between mutants and NILs is that the genetic
variation present in the NILs is the result of adaptations to local environmental variables. Seeds of all genotypes were harvested from plants grown under various light intensities, photoperiod, temperatures, nitrate and phosphate concentrations and seed performance was analysed by after-ripening requirement to release seed dormancy, seed longevity and germination under several stress conditions. In this paper we show that interactions between parental environment and genotype clearly affect plant and seed performance in Arabidopsis.

Materials and Methods

Plant materials

The Arabidopsis thaliana (Arabidopsis) accessions Landsberg erecta (Ler-0, Ler), Columbia (Col-0, Col) and other genotypes with the Ler and Col genetic backgrounds were used in this study. NIL DOG1-Cvi (Cape Verde Islands), NIL DOG2-Cvi, NIL DOG3-Cvi, NIL DOG6-Kas-2 (Kashmir), NIL DOG22-An-1 (Antwerpen) (Alonso-Blanco et al., 2003; Bentsink et al., 2010), and the dog1-1 mutant (Bentsink et al., 2006) are lines with a Ler genetic background, whereas dog1-3 (SALK 000867, T-DNA insertion in the promoter region of DOG1) (Bentsink et al., 2006), cyp707a1-1, cyp707a2-1 (Kushiro et al., 2004) and the Atnced6-Atnced9 double mutant (Lefebvre et al., 2006) are lines with a Col genetic background.

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. Upon the start of flowering, plants were transferred to the various environmental conditions (Table 1), for each condition three biological replicates containing five plants per replicate.

Plants that were known to flower earlier (NIL DOG2 and NIL DOG22) were planted 5 days later in order to synchronize the flowering. In case individual plants had already started flowering, those flowers and siliques were removed to make sure all the seeds developed under the specific environmental conditions. Due to space limitation in the growth compartments each environment was performed as an independent experiment containing the control condition, except for the second nitrate and temperature experiment as these were performed at the same time and share, therefore, the control. All experiments
at each growth condition were executed twice (first and second growth), for a robust confirmation of the phenotypes.

**Plant phenotyping**

Plant height, number of siliques per plant and number of seeds per silique were scored for all three replicates. To investigate the number of seeds per silique and seed size, flowers that had opened at day 10 after the start of flowering were tagged and corresponding seeds were harvested. When the majority of siliques had turned yellow, the plants were stopped watering and 7 days later dry mature seeds were harvested (Huang *et al.*, 2014). These criteria have been used for harvesting seeds in all the environments. The number of seeds was determined by taking photographs of the seeds on white filter paper (20.2 x 14.3 cm white filter paper, Allpaper BV, Zevenaar, The Netherlands, http://www.allpaper.nl) using a Nikon D80 camera fixed to a repro stand with a 60mm macro objective. The camera was connected to a computer with Nikon Camera Control Pro software version 2.0. Clustering of seeds was prevented as much as possible. The photographs were analyzed using ImageJ (http://rsbweb.nih.gov/ij/) by combining colour thresholds ($Y_{100-255}$, $U_{0-85}$, $V_{0-255}$) with particle analysis.

**Seed phenotyping**

Seeds were harvested as a bulk from five plants. Seeds were weighed with an AD-4 autobalance (PerkinElmer, Inc.). Single seed weight was determined by weighing around 5 mg of seeds, divided by the number of the weighed seeds, and converted to 1000-seed weight by multiplying with 1000.

Germination experiments were performed as described previously (Joosen *et al.*, 2010). In brief, two layers of blue germination paper were equilibrated with 48 ml demineralized water in plastic trays (15 x 21 cm). Six samples of approximately 50 to 150 seeds were spread on wetted papers using a mask to ensure accurate spacing. Piled up trays were wrapped in a closed transparent plastic bag. The experiment was carried out in a 22°C incubator under continuous light (143 µmol m$^{-2}$ s$^{-1}$). Pictures were taken twice a day for a period of 6 days using the same camera and software as described for number of seeds.

Germination was scored using the Germinator package (Joosen *et al.*, 2010). To quantify seed dormancy (DSDS50: days of seed dry storage required to reach 50% germination), germination tests were performed weekly until all seed batches had germinated for more than 90%. A generalized linear model with a logit link as described by Hurtado *et al.* (2012) was adapted to calculate DSDS50. Germination data were adjusted by choosing $n = 100$ and fitted as one smooth curve per line. The observed germination proportion was re-interpreted as having observed y ‘successes’ in n binomial trials (e.g.
75% germinated means y = 75 out of 100 possible ‘trials’). DSDS50 is the closest time point to where a horizontal line at y = 50 crosses the fitted curve.

Germination under stress conditions was performed on fully after-ripened seeds. Stress conditions were: temperature stress (10°C, 30°C); osmotic stress (-0.8 MPa mannitol; Sigma-Aldrich), salt stress (125 mM NaCl; Sigma-Aldrich), ABA stress (0.2 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 artificial aging test was performed by incubating seeds above a saturated ZnSO₄ solution (40°C, 85% relative humidity) in a closed tank with circulation for 5 days (ISTA, 2012). In the accelerated aging method (ISTA, 2012) and in our artificial aging method the seeds are constantly incubated in the same relative humidity combined with a warm temperature. The accelerated aging method of ISTA uses near 100% relative humidity, whereas we used 85% relative humidity. Then the seeds were taken out and germinated on demineralized water as described previously.

**Germination parameters**

Maximum germination ($G_{\text{max}}$) values were extracted from the germination assay using the Germinator package (Joosen *et al*., 2010). $G_{\text{max}}$ is the final germination percentage at the end of the germination assay. For germination in demineralized water (control), and germination at 10°C the $G_{\text{max}}$ of most genotypes reached 100%. Therefore, to better distinguish the small differences between genotypes, the rate of germination ($t_{50}$: the time required to reach 50% germination of the total number of germinated seeds) was also used for data analysis.

**Nitrate, phosphate and phytate determinations**

To measure nitrate, phosphate and phytate 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 min 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 an ion 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
standards of nitrate, phosphate and phytate were: NaNO\textsubscript{3} (Merck), Na\textsubscript{2}HPO\textsubscript{4}.2H\textsubscript{2}O (Merck) and Na(12)-IP6 IP6 (Sigma-Aldrich), respectively.

**Data analysis**

All data (both first and second growth) analysis was done in the statistical programming environment R 3.0.0:

*Integrated analysis of all factors contributing to plant and seed performance:* All data was analysed together. For comparison between traits each trait dataset was normalized to a scale of 0-100. Analysis of variance (ANOVA) using linear models was used to determine significance of the different environmental variables one by one.

*Integrated analysis of the effect of seed maturation environments on each plant and seed performance:* The dataset was split up by environmental factors as shown in Table 1. For data generated in each environment a linear model was fitted to determine the significance of the variable within the set. This was done over all genotypes. The significance threshold was adjusted for multiple testing by using significance 0.05 dividing the number of test (95) (\(P=0.000526\)).

*Trait by trait correlation/significance of plant and seed performance:* All data was used for this investigation. Pearson correlation was calculated for all trait pairs and significance was determined by linear regression.

*Genotype by environment interactions:* Data was split by environmental factors as described in Table 1. Genotype by environment interaction was determined by ANOVA using a linear model (trait~environment*genotype). Boxplots were generated by the standard R boxplot function, using the same linear model and data as use in the ANOVA as input.

**Results**

To identify the parental environment with the most dominant effect on seed performance, the different genotypes from flowering onwards were grown in the environments listed in Table 1. Twelve genotypes were used, including two wild type accessions Landsberg erecta (Ler), Columbia (Col-0), five near isogenic lines (NILDOG1, NILDOG2, NILDOG3, NILDOG6, NILDOG22) that are known to affect seed dormancy (Bentsink et al., 2010), as well as two mutants that are affected in the DOG1 gene (dog1-1 and dog1-3) and three mutants of ABA biosynthesis and catabolism genes (cyp707a1-1, cyp707a2-1 and nced6 nced9). Here, seed performance refers to the capacity of seeds to germinate under varying environmental conditions. Several seed germination traits were determined, including seed dormancy (DSDS50), seed longevity, and germination under stress conditions (i.e. high and low temperatures, osmotic stress, salt stress and ABA stress conditions).
Table 1. Environmental conditions before and after the start of flowering*.  

<table>
<thead>
<tr>
<th>Environmental factors</th>
<th>Before flowering</th>
<th>After flowering</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Light Intensity</strong></td>
<td><strong>Standard (SL)</strong></td>
<td><strong>Low (75 µmol m⁻² s⁻¹) (LL)</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Standard (150 µmol m⁻² s⁻¹), (SL)</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>High (300 µmol m⁻² s⁻¹), (HL)</strong></td>
</tr>
<tr>
<td><strong>Photoperiod</strong></td>
<td><strong>Long Day (LD)</strong></td>
<td><strong>Short Day (8h daylength) (SD)</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Long Day (16h daylength), (LD)</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Continuous Light (CL)</strong></td>
</tr>
<tr>
<td><strong>Temperature</strong></td>
<td>20°C</td>
<td>15°C</td>
</tr>
<tr>
<td></td>
<td>20°C</td>
<td>20°C</td>
</tr>
<tr>
<td></td>
<td>25°C</td>
<td>25°C</td>
</tr>
<tr>
<td><strong>Nitrate</strong></td>
<td>5 mM (N5)</td>
<td>0 mM (N0)</td>
</tr>
<tr>
<td></td>
<td>5 mM (N5)</td>
<td>20 mM (N20)</td>
</tr>
<tr>
<td><strong>Phosphate</strong></td>
<td>0.5 mM, 20°C (P0.5_20°C)</td>
<td>0.01 mM, 20°C (P0.01_20°C)</td>
</tr>
<tr>
<td></td>
<td>0.5 mM, 20°C (P0.5_20°C)</td>
<td>0.5 mM, 20°C (P0.5_20°C)</td>
</tr>
<tr>
<td></td>
<td>3 mM, 20°C (P3_20°C)</td>
<td>3 mM, 20°C (P3_20°C)</td>
</tr>
<tr>
<td></td>
<td>0.5 mM, 25°C (P0.5_25°C)</td>
<td>0.01 mM, 25°C (P0.01_25°C)</td>
</tr>
<tr>
<td></td>
<td>0.5 mM, 25°C (P0.5_25°C)</td>
<td>0.5 mM, 25°C (P0.5_25°C)</td>
</tr>
<tr>
<td></td>
<td>3 mM, 25°C (P3_25°C)</td>
<td>3 mM, 25°C (P3_25°C)</td>
</tr>
</tbody>
</table>

* After the start of flowering the plants were transferred to different parental conditions. For each condition the abbreviation that is used in Figure 1-5 has been indicated within brackets. Standard light intensity (SL), Long day (LD), 20°C, 5 mM nitrate (N5), 0.5 mM phosphate (P0.5) was regarded as control condition.

The seed performance phenotypes observed in control condition confirmed those that had been reported previously (Fig. S1) (Alonso-Blanco et al., 2003; Kushiro et al., 2004; Bentsink et al., 2006; Lefebvre et al., 2006; Bentsink et al., 2010). Since the environment has both direct and indirect effects on seed performance we also monitored several plant phenotypes, such as the time that is required for seed maturation, plant height, number of siliques per plant, number of seeds per silique, seed size and seed weight.
The effect of the parental environment on the seed reproductive period

Low light intensity and short day extended the seed reproductive period with approximately 10 days (Fig. 1). In contrast, increased light intensity and extended photoperiod had no influence on the length of the seed reproductive period.

Low temperature (15°C) retards plant growth and, as a result, extends the reproductive period (Fig. 1). At 15°C, all genotypes required almost one month extra to complete their life cycle as compared to 20°C, whereas at higher temperature (25°C) the reproductive period was shortened by 12 days.

Different nitrate and phosphate concentrations had no effect on the reproductive period. In addition, the combination of phosphate and high temperature shortened the reproductive period as for high temperature alone. This confirmed that changes in phosphate level do not affect the length of the reproductive period.

![Figure 1. Plant reproductive periods in different environments as presented in Table 1. The average value of first and second growth was shown. Error bars show standard deviation. Low light (LL); standard light (SL); high light (HL), short day (SD); long day (LD), continuous light (CL), nitrate 0 mM (N0), nitrate 5 mM (N5), nitrate 20 mM (N20), phosphate 0.01 mM at 20°C (P0.01_20°C), phosphate 0.5 mM at 20°C (P0.5_20°C), phosphate 3 mM at 20°C (P3_20°C), phosphate 0.01 mM at 25°C (P0.01_25°C), phosphate 0.5 mM at 25°C (P0.5_25°C), phosphate 3 mM at 25°C (P3_25°C). SL, LD, 20°C, N5, P0.5 are control conditions.]
Generalized effects of the parental environments on plant and seed performance

Overall we have phenotyped 12 different genotypes (three biological replicates each) for 18 traits in 13 different environments and all these experiments have been performed twice. In order to assess and compare the importance of the different environmental factors on the phenotypes investigated, we performed statistical analysis on all data generated.

Relationships between traits

A correlation matrix was generated for all pairs of measured traits to investigate associations between the characterized traits (Fig. 2, Table S2). The plant performance traits plant height and number of siliques per plant showed a strong and highly significant positive correlation. Plant height was also strongly correlated with seed weight, and, to a lesser extent, with seed size. Seed weight and seed size were strongly correlated. In general stress germination traits correlated with each other, especially germination in mannitol and salt, probably because both confer osmotic stress.

The effect of the parental environmental factors

We used linear models/ANOVA to investigate the overall variance caused by the different parental environments. This analysis revealed that in general the effect of the genotype was the most pronounced ($P < 1 \times 10^{-200}$) (Table 2), with a very prominent contribution of the genetic background (Ler or Col) of the genotypes ($P < 1 \times 10^{-113}$). Also the effect of the parental environment was very significant ($P < 1 \times 10^{-157}$). Of the environmental factors, temperature had the most significant effect on the traits measured ($P < 1 \times 10^{-60}$; Table 2). A linear model was used to determine the significance of the different environments on each plant as well as on the seed traits (Table 3). Temperature played a dominant role in both plant and seed traits (as was also shown in Table 2), whereas light signals (light intensity and photoperiod) had more impact on plant traits. Nitrate mildly affected some of the plant and seed traits while phosphate had even less influence on those traits. To illustrate the direction of the effect of the parental environment, we have presented plant and seed performances in Fig. 3 and Fig. 4, respectively. Only the data for Ler and Col are presented since most of the effects were similar in all genotypes and the largest differences were caused by the genetic background. The significance of all the genotypes for both growths is shown in Table 3. The effects that are different from the control treatment in both independent growths (t-test, $P < 0.05$) are presented in Fig. 3 and Fig. 4.
Figure 2. Trait by trait correlation/significance of plant and seed performance. The linear model was used to calculate all pairwise correlations between plant and seed performance traits. In the red/blue coloured area, rectangles represent Pearson correlation coefficient $r$ values (see correlation colour key). In the yellow/grey coloured area, rectangles represent $-\log (P\text{-values})$ of the Pearson correlation coefficients (see Significance colour key) and empty rectangles represent not significant ($P\text{-values}$ greater than 0.01). DSDS50 (days of seed dry storage until 50% of germination) represents dormancy levels. Longevity is measured by artificial aging (40°C, 85% relative humidity). $G_{\text{max}}$ is the final germination percentage at the end of the germination assay. $t_{50}$ is the rate of germination. NA: not available.
Table 2. Integrated analysis of all factors contributing to plant and seed performance.

<table>
<thead>
<tr>
<th>Factors</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>$&lt;1 \times 10^{-200}$</td>
</tr>
<tr>
<td>Environmental summary*</td>
<td>$&lt;1 \times 10^{-157}$</td>
</tr>
<tr>
<td>Background</td>
<td>$&lt;1 \times 10^{-113}$</td>
</tr>
<tr>
<td>Temperature</td>
<td>$&lt;1 \times 10^{-60}$</td>
</tr>
<tr>
<td>Light intensity</td>
<td>$&lt;1 \times 10^{-49}$</td>
</tr>
<tr>
<td>Photoperiod</td>
<td>$&lt;1 \times 10^{-27}$</td>
</tr>
<tr>
<td>Phosphate</td>
<td>$&lt;1 \times 10^{-25}$</td>
</tr>
<tr>
<td>Nitrate</td>
<td>$&lt;1 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

* Environmental summary is the combination of the five environmental factors. For every factor the $P$-value is reported to indicate significance.

The effect of light intensity on plant and seed performance

Light intensity affected all the plant phenotypes significantly (Table 3). In high light intensity plants grew taller (Fig. 3A) and produced more siliques per plant (Fig. 3B). Low light intensity significantly decreased the number of seeds per silique (Fig. 3C). High light intensity resulted in heavier (Fig. 3D) and larger seeds (Fig. 3E). High light intensity also had a positive effect on germination percentage in ABA (Fig. 4A), germination rate in 10°C (Fig. 4B) and seed longevity (Fig. 4C), as measured by artificial aging.

The effect of photoperiod on plant and seed performance

Both photoperiod and light intensity are important light signals to plant performance, but they play distinct roles. Short days decreased the number of seeds per silique (Fig. 3C) while continuous light resulted in heavier (Fig. 3D) and larger seeds (Fig. 3E). These results were in agreement with Contreras et al. (2008). Contrary to light intensity, photoperiod did not have any significant effect on seed performance (Table 3).

The effect of temperature on plant and seed performance

Low temperature (15°C) during seed maturation resulted in yield increases. Plants had more siliques (Fig. 3B) that contained heavier (Fig. 3D) and larger seeds (Fig. 3E). However, it is worth noting that the quality of these seeds was lower than that of the control, which is especially reflected in the decreased seed longevity (Fig. 4C), and
decreased germination in salt (Fig. 4D) and in mannitol (Fig. 4E). The low seed maturation temperature also slowed the germination rate at 22°C (Fig. 4F).

**Table 3.** Integrated analysis of the effect of seed maturation environments on plant and seed performance*.

<table>
<thead>
<tr>
<th></th>
<th>Light Intensity</th>
<th>Photoperiod</th>
<th>Temperature</th>
<th>Nitrate</th>
<th>Phosphate</th>
<th>Phosphate x Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant height</td>
<td>7.60</td>
<td>11.38</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Silique per plant</td>
<td>24.63</td>
<td>24.79</td>
<td>10.00</td>
<td>4.88</td>
<td>6.35</td>
<td>n.s.</td>
</tr>
<tr>
<td>Seeds per silique</td>
<td>15.87</td>
<td>33.37</td>
<td>10.01</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Seed size</td>
<td>5.75</td>
<td>39.01</td>
<td>32.93</td>
<td>n.s.</td>
<td>4.08</td>
<td>n.s.</td>
</tr>
<tr>
<td>Seed weight</td>
<td>42.54</td>
<td>15.84</td>
<td>8.61</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>DSDS50</td>
<td>n.s.</td>
<td>n.s.</td>
<td>7.39</td>
<td>3.65</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Longevity</td>
<td>12.05</td>
<td>n.s.</td>
<td>16.78</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>$G_{\text{max}}$ 22°C</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>$t_{50}$ 22°C</td>
<td>n.s.</td>
<td>n.s.</td>
<td>27.56</td>
<td>6.05</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>$G_{\text{max}}$ 10°C</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>$t_{50}$ 10°C</td>
<td>5.67</td>
<td>n.s.</td>
<td>8.02</td>
<td>5.28</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>$G_{\text{max}}$ 30°C</td>
<td>n.s.</td>
<td>n.s.</td>
<td>13.77</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>$G_{\text{max}}$ mannitol</td>
<td>n.s.</td>
<td>n.s.</td>
<td>11.63</td>
<td>4.09</td>
<td>3.68</td>
<td>n.s.</td>
</tr>
<tr>
<td>$G_{\text{max}}$ salt</td>
<td>n.s.</td>
<td>n.s.</td>
<td>11.88</td>
<td>n.s.</td>
<td>4.85</td>
<td>n.s.</td>
</tr>
<tr>
<td>$G_{\text{max}}$ ABA</td>
<td>15.06</td>
<td>n.s.</td>
<td>6.64</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Nitrate content</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>5.72</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>Phosphate content</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>8.98</td>
<td>n.s.</td>
</tr>
<tr>
<td>Phytate content</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>23.39</td>
<td>8.08</td>
</tr>
</tbody>
</table>

* -10log ($P$) values demonstrate significance levels. n.a.: not available; n.s.: not significant ($P<0.000526$). DSDS50 represents dormancy levels. Longevity is measured by artificial aging (40°C, 85% relative humidity). $G_{\text{max}}$ is the final germination percentage at the end of the germination assay. $t_{50}$ is the rate of germination.

**The effect of nutrition on plant and seed performance**

The effect of both nitrate and phosphate and a combination of phosphate regimes and high temperature on plant and seed performance was studied. Plants grown in the higher nitrate regime (20 mM) produced more siliques (Fig. 3B). With respect to seed performance, low nitrate (0 mM) decreased germination rate (Fig. 4B) and decreased germination in mannitol (Fig. 4E) but higher nitrate did not have a significant effect.
Phosphate levels correlated positively with the number of siliques per plant (Fig. 3B) (Zhao et al., 2008; Dick et al., 2011). Increasing phosphate content increased germination in stress conditions (Fig. 4D and 4E) at 20°C. Phytate is the main storage form of phosphate in dry seeds, and the level of phytate increased in the high phosphate maturation environment accordingly (Fig. 4G).

**Figure 3.** General effect of the seed maturation environment on plant performance. Plant performance of both Landsberg *erecta* (Ler) and Columbia (Col) are presented: (A) plant height (cm), (B) number of siliques per plant, (C) number of seeds per silique (D) weight of 1000 seeds (mg), (E) seed size (mm$^2$) for light intensity (low light (LL); standard light (SL) and high light (HL)), photoperiod (short day (SD); long day (LD) and continuous light (CL)), temperature (15, 20 and 25°C), nitrate concentrations (N0, N5 and N20) and phosphate concentrations (P0.01_20°C, P0.5_20°C, P3_20°C), respectively. Only the results that were significant ($P<0.000526$; Table 3) and repeatable in both growths are presented here. Averages of three replicates are displayed. Error bars show standard errors.

**Genotype-specific effects of the parental environment on seed performance**

**Genotype by environment interactions**

Maturation environments have a noteworthy influence on seed dormancy levels, as well as on other plant and seed performance traits. However, several highly significant genotype by environment (GxE) interactions suggest that the phenotypic plasticity varied among the 12 genotypes tested. All the significant ($P<0.001$) GxE interactions for the parental maturation environments are listed in Table 4. To visualize this GxE effect we have shown the dormancy levels (DSDS50) for the nitrate environment (Fig. 5). Genotypes with higher primary dormancy levels display higher plasticity in the different nitrate
environments. Thus, specific genetic regions (NILs) or genes (mutants) are the likely causal factors of higher plasticity. Furthermore, we see mainly an effect of reduced nitrate (0 mM) and not that of increased nitrate. Apparently, the nitrate response is saturated between N20 and N5. All of the significant GxE interactions affecting plant and seed performances in the different environments are shown in Fig. S2. To explore this in more detail, we focus on genotype-specific effects in the following section.

**Figure 4.** General effect of the seed maturation environment on seed performance. Seed performance of both Landsberg erecta (Ler) and Columbia (Col) is presented. (A) germination in ABA (0.2 µM), (B) t\textsubscript{50} for germination at low temperature (10°C), (C) germination after artificial aging (40°C, 85% relative humidity), (D) germination in salt (125 mM NaCl), (E) germination in mannitol (-0.8 MPa), (F) t\textsubscript{50} for germination at 22°C. (G) phytate content in seeds (mg g\textsuperscript{-1} seeds) for light intensity (low light (LL); standard light (SL) and high light (HL)), photoperiod (short day (SD); long day (LD) and continuous light (CL)), temperature (15, 20 and 25°C), nitrate concentrations (N0, N5 and N20) and phosphate concentrations x temperature (P0.01_20°C, P0.5_20°C, P3_20°C, P0.01_25°C, P0.5_25°C, P3_25°C), respectively. Only the results that were significant (P<0.000526; Table 3) and repeatable in both growths are presented here. Averages of three replicates are displayed. Error bars show standard errors.

**Effect of the parental environment on seed dormancy and longevity**

Low light intensity increased seed dormancy of NILDOG3 and NILDOG6 (Fig. 6A). Germination percentage after artificial aging decreased in low light intensity, indicating a negative correlation with seed dormancy for NILDOG3 and NILDOG6 (Fig. 6). However, the response of seed longevity to light was much more pronounced than that of dormancy. Light intensity significantly affected seed longevity for all genotypes tested (Fig. 6B and Fig. S2).
Our results show that the low maturation temperature significantly increased dormancy in NILDOG1 (Fig. 6C), but also in the other genotypes (Fig. S2). This can be explained by the functional DOG1 Ler and Col alleles that are present in these lines, which is supported by the lack of response in the dog1 mutants (Fig. S2). Also for temperature we identified a negative correlation between seed dormancy and seed longevity (Fig. 6C and 6D).

Table 4. Significant genotype by environment interactions affecting plant and seed performance in all five environments*.

<table>
<thead>
<tr>
<th>Environment</th>
<th>Plant/Seed performance</th>
<th>GxE P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Light Intensity</strong></td>
<td>DSDDS50</td>
<td>2.5$^{10}$</td>
</tr>
<tr>
<td></td>
<td>$G_{\text{max}}$ 10°C</td>
<td>3.3$^{23}$</td>
</tr>
<tr>
<td></td>
<td>$G_{\text{max}}$ 22°C</td>
<td>4.7$^{15}$</td>
</tr>
<tr>
<td></td>
<td>$G_{\text{max}}$ mannitol</td>
<td>2.2$^{08}$</td>
</tr>
<tr>
<td></td>
<td>$G_{\text{max}}$ salt</td>
<td>4.0$^{09}$</td>
</tr>
<tr>
<td></td>
<td>Seed weight</td>
<td>3.8$^{07}$</td>
</tr>
<tr>
<td><strong>Photoperiod</strong></td>
<td>DSDDS50</td>
<td>2.3$^{09}$</td>
</tr>
<tr>
<td></td>
<td>Seed weight</td>
<td>2.2$^{07}$</td>
</tr>
<tr>
<td><strong>Temperature</strong></td>
<td>DSDDS50</td>
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</tr>
<tr>
<td></td>
<td>$G_{\text{max}}$ 22°C</td>
<td>3.1$^{09}$</td>
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<td>$G_{\text{max}}$ 30°C</td>
<td>2.7$^{04}$</td>
</tr>
<tr>
<td></td>
<td>$G_{\text{max}}$ mannitol</td>
<td>7.6$^{04}$</td>
</tr>
<tr>
<td></td>
<td>$G_{\text{max}}$ salt</td>
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</tr>
<tr>
<td></td>
<td>Longevity</td>
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<tr>
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<td>$G_{\text{max}}$ 10°C</td>
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<td>$G_{\text{max}}$ 22°C</td>
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<tr>
<td></td>
<td>$G_{\text{max}}$ ABA</td>
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<td>2.2$^{06}$</td>
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<tr>
<td><strong>Phosphate</strong></td>
<td>$G_{\text{max}}$ 30°C</td>
<td>1.4$^{04}$</td>
</tr>
<tr>
<td></td>
<td>$G_{\text{max}}$ salt</td>
<td>6.0$^{04}$</td>
</tr>
</tbody>
</table>

* GxE, genotype by environment interaction. DSDDS50 represents dormancy levels. Longevity is measured by artificial aging (40°C, 85% relative humidity). $G_{\text{max}}$ is the final germination percentage at the end of the germination assay.
Nitrate dosage, particularly low nitrate (0 mM) during silique formation, increased the dormancy levels of NILDOG1 and cyp707a1-1 (Fig. 6E), but not of cyp707a2-1 (Fig. S2). Thus, the loss of function mutation in CYP707A2 leads to a defective response to nitrate and therefore no increase in dormancy.

Figure 5. Genotype by environment interactions for seed dormancy behaviour after seed maturation in different nitrate regimes. The Boxplot presents dormancy levels (DSDS50) of 12 genotypes in three nitrate environmental conditions (N0, N5 and N20). The genotype by environment interaction is significant ($P=4.03^{-18}$).

The effect of the parental environment on germination in stress conditions

In general, high light intensity, continuous light, high temperature (25°C), high nitrate and high phosphate resulted in higher germination percentage under stress (Fig. S2). Germination behaviour in mannitol and salt were positively correlated as described above (Fig. 2; Table S2; $P=4.05^{-96}$). The nced6 nced9 double mutant that produces less ABA in its seeds (Lefebvre et al., 2006) showed the most distinguishable germination phenotype, germinating to approximately 100% in both salt and mannitol, irrespective of the
maturation environment (Fig. 7). Meanwhile, *cyp707a2-1* was always sensitive to stress conditions, and to a higher extent than *cyp707a1-1* (Fig. 7).

**Figure 6.** Dormancy (DSDS50) and longevity levels (germination after artificial aging) of seeds matured in different light intensity (A, B), temperature (C, D) and nitrate concentrations (E, F). Averages of three replicates are presented. Error bars show standard errors. Asterisks indicate significant differences between treatment and control of each genotype (*P*<0.05).
Figure 7. Mannitol (A) and salt (B) stress germination of an ABA biosynthesis double mutant (nced6 nced9) and catabolic mutants (cyp707a1-1 and cyp707a2-1) grown under different environments: light intensity (low light (LL); standard light (SL) and high light (HL)), nitrate concentrations (N0, N5 and N20), phosphate concentrations (P0.01_20°C, P0.5_20°C, P3_20°C) and phosphate concentrations x temperature (P0.01_20°C, P0.5_20°C, P3_20°C, P0.01_25°C, P0.5_25°C, P3_25°C). Averages of three replicates are presented. Error bars show standard errors. Asterisks indicate significant differences between treatment and control of each genotype ($P<0.05$).
Discussion

Knowledge about the effect of the parental environment on seed performance provides more insight in fundamental principles of how the environment may influence the fitness of a species, as measured by fruit and seed yield, and seed performance. Such knowledge will not only help to predict seed performance but also assist in the improvement of breeding programs and seed production by indicating the best location, season, and soil type to increase yield and seed quality. By using 12 genotypes and 13 different seed maturation environments, our study provides a very detailed insight into the effect of the environment, genotype and the genotype by environmental interactions on plant and seed performance.

Genotype by environment interactions

The usefulness of studying different genotypes became apparent from the fact that clear genotype-specific effects were observed. Most obvious is the effect of the genetic background (Table 2, \(P<1\times10^{-13}\)). Ler and Col genotypes responded with a similar trend to the changes in the environment but Col plants are taller, produce more siliques per plant and are generally more stress tolerant (higher germination in ABA and higher germination after artificial aging) (Fig. 3 and Fig. 4). Furthermore, strong GxE interactions were observed (Table 4, Fig. 8). These interactions show that certain genotypes respond differently to specific environmental cues, as discussed below in detail. Similar effects were, to some extent, also shown by Munir et al. (2001) who reported a significant genotype by maternal photoperiod interaction for Calver-0 and Tacoma-0 recombinant inbred lines in long day and short day conditions. A similar interaction was found in this population for quantitative trait loci (QTL) identified on chromosome 3 which had a significantly stronger effect on fitness in June-dispersed seeds than in November-dispersed seeds (Huang et al., 2010). The role of the maternal photoperiod on GxE interactions was also shown for Ler and Col when combining photoperiod and temperature (Donohue et al., 2007; Donohue, 2009). Contrary to these studies we chose a more comprehensive approach by using a large set of genotypes (12), measuring 5 plant and 13 seed performance traits after plant exposure to different light intensities, photoperiods, temperatures and nitrate and phosphate concentrations.
Figure 8. Summarizing model of parental environmental effects on seed dormancy and longevity. Red and blue lines represent the environmental conditions which increase or decrease the trait level, respectively. Bold lines show the negative correlation between dormancy and longevity.

Photoperiod, phosphate and phosphate x temperature combination did not have significant effects on seed dormancy and longevity while light intensity, temperature and nitrate showed clear genotype-specific responses (Fig. 8). Low light conditions increased dormancy in NILDOG3 and NILDOG6, whereas temperature mainly affected NILDOG1 and cyp707a1-1 (Fig. 6 and Fig. 8). It remains unclear how light intensities can affect NILDOG3 and NILDOG6 since the underlying genes have not been cloned yet. For DOG1 it is known that low temperature during seed maturation increases its expression and thereby seed dormancy (Chiang et al., 2011; Kendall et al., 2011; Nakabayashi et al., 2012). This effect of seed maturation temperatures on seed dormancy levels has also recently been reported by Huang et al. (2014). Low nitrate conditions also specifically increased seed dormancy in NILDOG1 and cyp707a1-1, as well as in the background accessions Ler and Col (Fig. 8). The precise effect of the environment on DOG1 and CYP707A1 remains to be investigated. However, both DOG1 expression and ABA levels in buried seeds increase in winter (Footitt et al., 2011) and CYP707A1 is probably required for the ABA breakdown. This agrees with the observed higher ABA levels in NILDOG1 and cyp707a1-1 in low nitrate and low temperature conditions (Fig. S3, see ‘Supplementary data’ section for the ABA extraction and detection method).
ABA metabolism and signalling are responsive to many important developmental processes and environmental cues, which makes it a key regulator of growth in changing environments (Nambara & Kuchitsu, 2011). These physiological processes are primarily regulated by ABA maintenance, through fine-tuning of the rates of de novo biosynthesis and catabolism (Saito et al., 2004). The ABA biosynthesis defective nced6 nced9 double mutant was far less sensitive to changing environments, suggesting a role for de novo ABA synthesis during imbibition in these conditions. The expression of the ABA catabolic gene CYP707A2 was induced dramatically after 6 hours of imbibition whereas CYP707A1 did not peak at the early stages of germination. Therefore, CYP707A2 is probably more effective in the upregulation of ABA degradation during germination (Liu et al., 2009). This hypothesis is supported by the observation that cyp707a2-1 was always more sensitive to germination under stress (mannitol and salt) than cyp707a1-1 (Fig. 7 and Fig. S2).

The responses discussed above are direct effects of the environment on seed development and maturation; however, there might also be indirect responses. The increased length of the reproductive period at low temperature (Fig. 1) can be the cause of the heavier (Fig. 3D) and larger (Fig. 3E) seeds. Seeds are on the plant longer, thus allowing increased nutrient translocation and reserve accumulation. Effects on seed performance in low light intensity, short days and 15°C seem to be direct since the reproductive period was extended by 10-15 days for all three treatments but seed performance responses were different among the three environments (Fig. 4).

**Negative correlation between seed dormancy and longevity**

The negative correlation that we found between seed dormancy and seed longevity is in agreement with the recently reported negative correlation of seed dormancy and seed longevity QTLs in Arabidopsis (Nguyen et al., 2012). This surprising finding was in contradiction with all earlier reported work. We hypothesized that this observation was linked to the fact that we used natural variation for our studies, in contrast to the earlier work that was all based on mutant analyses in Arabidopsis (i.e. leafy cotyledon1 (lec1), abscisic acid intensive3 (abi3), transparent testa (tt), aberrant testa shape (ats), delay of germination1 (dog1) and the green seed mutant (Ooms et al., 1993; Debeaujon et al., 2000; Clerkx et al., 2003; Clerkx et al., 2004; Bentsink et al., 2006; Sugliani et al., 2009). A negative correlation between seed dormancy and seed longevity, confirming our hypothesis, and a role for environmental adaptation has recently been described for *Eruca sativa* (Barazani et al., 2012; Hanin et al., 2013). These authors studied *E. sativa* plants that are distributed in Israel in a narrow geographic area, along different habitats ranging from arid-dry environments to more mesic habitats and found that dormancy increased with increasing aridity and that seed longevity decreased along this gradient. Moreover, De Cauwer et al. (2014) have demonstrated variation for dormancy and longevity in natural
populations of *Galinsoga parviflore* and *G. quadriradiate*. Also in this weed the less dormant species showed the highest longevity.

A novel aspect of our current work is that we can affect this correlation by changing the seed maturation environment. Increasing light intensity and temperature decreased primary dormancy and increased seed longevity, as measured by artificial aging (Figs. 6 and 8). This may refer to the selective pressure that has shaped natural variation for these traits and thereby their evolutionary path. This is in line with the fact that seed dormancy is an adaptive trait (Bewley, 1997) that displays strong adaptive plasticity to geographic locations and seasonal conditions (Donohue *et al.*., 2005a). Based on our data it is unclear which of the two traits (dormancy or longevity) is under selective pressure since there might be a trade-off between them. Identifying this trade-off mechanism will require more in-depth studies but we speculate that this trade-off represents an adaptive mechanism to maximize fitness under contrasting environments. Arabidopsis growing in temperate climates (mild and wet summer conditions) are best synchronized with dormancy cycling mechanisms. These mechanisms involve de- and rehydration cycles, allowing, at intervals, activation of repair mechanisms to counteract aging-related damage, such as the repair of damaged DNA, proteins and mobilization of proteins (Gamboa-deBuen *et al.*, 2006; Kranner *et al.*, 2010; Rajjou *et al.*, 2012). In warmer and drier climates seeds will have to survive hot and dry conditions. Under these conditions, de- and rehydration cycles are absent and dormancy cycling may be less relevant for fitness. In this case the selective pressure could be on longevity rather than on dormancy. Thus, in high maternal temperature and light, dormancy levels are low because these climatic conditions (seasons) are associated with drought that does not allow germination until water is present. Hence, in this situation germination is controlled by the environment rather than by seed dormancy. The conditions that are encountered by the plant and seeds also depend on whether plants behave like summer or winter annuals, which in addition to germination phenology is determined by flowering time. Analyses of natural populations for which geographical and environmental data is available might provide more insight into the selective pressures that shaped natural variation for these important seed traits (Méndez-Vigo *et al.*, 2011).

**Transfer of knowledge to crops**

In the present study we have investigated the effect of single environments and only in one case the effect of a combined environment (phosphate and high temperature) which did not have any significant effect on either plant or seed performance (Table 3), whereas the single environments phosphate and temperature did have significant effects. In the field, environmental conditions are more complex. Plants often have to deal with combinatorial changes. For example, high light intensity is most likely accompanied by high temperature, whereas winter combines cold, short photoperiod and low radiation. Moreover, our analysis revealed that light intensity may significantly affect plant
performance. Doubling the light intensity increased plant height, number of siliques per plant, number of seeds per siliques, seed weight and seed size. Standard laboratory light intensities used in this and other studies (150 µmol m\(^{-2}\) s\(^{-1}\)) are still rather low compared to that of sunlight (in open field, 1500 - 2000 µmol m\(^{-2}\) s\(^{-1}\) on sunny days and 200 - 450 µmol m\(^{-2}\) s\(^{-1}\) on cloudy days at noon in summer in the Netherlands (Global radiation data in 2012 from De Kring – Bleiswijk, the Netherlands) (Mishra et al., 2012). Our study indicates that Arabidopsis benefits substantially from growth at high light intensities. Whether crop plants respond in a similar way remains to be investigated, as well as whether striving for higher sunlight levels has the same advantages. Furthermore, we see that increased photoperiod, especially continuous light enhances plant performance, which might be detrimental to some crops, for example, tomato (Velez-Ramirez et al., 2011). The research performed here in the model plant Arabidopsis provides directions for further investigations in crop species.

In general, a combination of low seed dormancy and high longevity is a desirable trait for crop species but also for seed conservation, although a certain level of dormancy may be required to prevent pre-harvest sprouting. Our study implies that manipulation of the growth conditions may be used to culture seeds with the required seed performance. Alternatively, monitoring the growth conditions during seed maturation may help to predict performance of the mature seed.

Conclusions

Our comparative analyses confirm and extend the notion that variable environmental conditions during seed development may result in variable plant and seed performance. Of all five parental environments analysed, temperature changes during seed maturation played a dominant role in both plant and seed performance, whereas light signaling (light intensity and photoperiod) had more impact on plant traits. Nitrate and phosphate displayed relatively mild effects on plant and seed performance. The observation that the different genotypes responded differentially to the environmental conditions indicates that different genetic and molecular pathways are involved in these responses.

Acknowledgement

We thank Dr. Eiji Nambara (University of Toronto) for providing cyp707a1-1, cyp707a2-1 seeds, Dr. Annie Marion-Poll (INRA) for providing the Atnced6 Atnced9 double mutant, Dr. Wilco Ligterink (Wageningen Seed Lab) for valuable discussions, Kerstin Gühl for ABA measurements, Diaan Jamar for nitrate, phosphate and phytate measurements, Dr. Jan E. Kammenga (Wageningen University) for his support.
Supplementary Material

Supplemental files can be downloaded from:
http://www.wageningenseedlab.nl/thesis/dsvidigal

Table S1. Element concentrations in the standard nutrient solution

Table S2. Trait by trait correlation/significance of plant and seed performance.

Figure S1. Seed dormancy levels and seed longevity levels of plants grown in control conditions (i.e. standard light, long days, 20°C, 5 mM nitrate and phosphate 0.5 mM phosphate).

Figure S2. Plant and seed performances of each genotype in five environments (light intensity, photoperiod, temperature, nitrate and phosphate).

Figure S3. ABA levels in freshly harvested seeds of Ler, NILDOGI, Col and cyp707a1-1 matured in low temperature and low nitrate compared with the control condition.

Supplemental material 1: ABA extraction and detection method.
References


Nakabayashi K.; Bartsch M.; Xiang Y.; Miatton E.; Pellengahr S.; Yano R.; Seo M.; Soppe W.J. (2012) The time required for dormancy release in Arabidopsis is determined by DELAY OF GERMINATION1 protein levels in freshly harvested seeds. The Plant Cell, 24, 2826-2838.


Chapter 3

Arabidopsis in the wild: the behaviour of seed dormancy during the seasons

Abstract

Climate changes play a central role in the adaptive life histories of organisms all over the world. In higher plants, these changes may impact seed performance, both during seed development and after dispersal. In order to inspect the plasticity of seed dormancy as a response to environmental variations, eight genotypes that are known to be affected in seed dormancy were grown during the four seasons of the year (summer, autumn, winter and spring) in the field. In order to determine the conditions that the plant experiences in the different seasons we have monitored soil temperature, air temperature and day length. Performance of seeds that had developed under natural conditions (field) was also compared with seeds from plants grown in controlled conditions (growth chamber at 20°C/18°C (day/night) under 16h photoperiod and 70% relative humidity). Life cycle, seed size and dormancy level were analysed. Our study indicates that, depending on the time of the year when plants are grown, seed performance varies. Plants grown during summer, when the days began to shorten and the temperature started to decrease, produced smaller seeds with deeper dormancy. Seed dormancy is largely controlled by the environment since the differences caused by the different seasons were larger than the differences among the genotypes. In addition, plants grown in a controlled environment produced larger seeds with lower dormancy than those grown in the field.

Vidigal D.S.*; He H.*; Hilhorst H.W.M.; Bentsink L. (*These authors contributed equally to this work)
Introduction

Seeds are part of the survival strategy of higher plants. Seeds allow the continuation of life after the mother plant senescence or is predated. Many types of plants, particularly the ephemerals, use seed dormancy as a survival strategy (Marcos Filho, 2005). Dormancy prevents seeds to germinate all at once after maturation and dispersal, thus avoiding the possible destruction of the species, in case of unpredictable unfavourable environments after germination (Bewley et al., 2013). Seed dormancy contributes to the adaptation of plants to their environment by optimizing the germination to the right period of the year. The level of dormancy (primary dormancy) in seeds is usually determined by several factors, such as genetic and the environmental factors operating during development and maturation (Gutterman, 2000; Fenner & Thompson, 2006; Hilhorst, 2011; Bewley et al., 2013). In the field seeds are commonly subjected to fluctuating temperatures which usually comprise low night temperatures and high temperatures during the day. These diurnal temperature fluctuations are frequently effective in dormancy breakage of many species (Bewley et al., 2013). Seed dormancy can also be released by after-ripening (AR), which occurs during storage of dry seeds during several months at a mild/warm temperature or by stratification, a low-temperature treatment (cold stratification) of imbibed seeds (Bewley, 1997; Bewley et al., 2013).

Arabidopsis thaliana (Arabidopsis) is an annual species which is spread across the northern hemisphere and grows in contrasting habitats along latitude, longitude and altitude. This geographic variation may influence the life cycle of natural populations, which can be divided into winter and spring annuals (Donohue, 2009). Winter annual life cycle occurs when seeds germinate in autumn and the seedlings or rosettes are maintained during the winter, after which, during the spring or beginning of summer the plants flower, and set and disperse seeds. Alternatively, the spring annual life cycle starts when seeds germinate in spring and grow into mature plants that flower, set seed and disperse their seeds in the same spring or summer (Donohue, 2009). The life cycle history of plants is important to the survival of the species because they need to avoid harsh environments and reproduce when the conditions are good enough for establishment and growth. This is especially important during germination and flowering time (Chiang et al., 2011).

Climate plays a central role in the adaptive life histories of organisms all over the world. Rutter & Fenster (2007) demonstrated that Arabidopsis populations worldwide exhibit adaptive differentiation in response to different climates. Such developmental responses to seasonal variation of the climate are important in understanding the evolutionary events of plant adaptation in nature (Donohue, 2009).

Environmental signals during seed development, maturation and after dispersal, such as photoperiod, temperature and drought stress, may influence the dormancy levels of the seeds at dispersal, affecting the requirements for dormancy to be relieved (Donohue, 2009; Walck et al., 2011). The role of day length, light quality, temperature, water and
nuts and nutrients in determining the degree of dormancy has been investigated in a wide range of species (Fenner, 1991; Hilhorst, 1995; Baskin & Baskin, 2014). The behaviour of the progeny can be affected by day length during the last stages of seed maturation (Gutterman, 2000). Germinability of some species can be higher for seeds produced under short days (Gutterman, 1973; Gutterman, 1978; Munir et al., 2001). However, Contreras et al. (2008) and He et al. (2014) did not find any significant effect of day length on seed performance.

Temperature during seed development and maturation is one of the most important determinants of seed germinability or seed dormancy in many species (Fenner, 1991; Biddulph et al., 2007; Llorens et al., 2008; Javaid et al., 2010; Kendall & Penfield, 2012; Huang et al., 2014; He et al., 2014). Seeds that develop at warmer temperatures are generally less dormant at maturity than those that develop at cooler temperatures as described for many species, including Beta vulgaris, Lactuca sativa, Amaranthus retroflexus, wild oat, Avena fatua (Fenner, 1991) and Arabidopsis (Kendall et al., 2011; Penfield & Springthorpe, 2012; Huang et al., 2014; He et al., 2014). However, elevated maternal temperatures can enhance primary dormancy in some species such as Syringa vulgaris and Syringa reflex (Juntila, 1973), Sisymbrium officinale (Burghardt et al., 2015), Xanthium pensylvanicum and Helianthus annuus (Baskin & Baskin, 2014). Recently, Huang et al. (2014) showed that in Arabidopsis both seed yield and dormancy were highly reduced by higher temperatures, but accessions showed a differential response, demonstrating that this temperature response is not solely an environmental effect. The natural genetic variation of the accessions also plays a role in seed performance phenology. This indicates that projected climate change may impact seed performance, but that the consequences will differ between species or within the same species. Insight in both the effect of the environmental conditions and the genetic basis is also important for understanding how seasonal dormancy may have evolved.

It has been shown that both field and laboratory experiments supply valuable knowledge about the regulation of germination and emergence in the field (Donohue, 2009). Here we investigated the plastic response of seed performance (seed dormancy and seed size) during all seasons of the year (spring, summer, autumn and winter) using a set of Near Isogenic Lines (NILs) that contain introgression fragments which are known to affect seed dormancy. We have also grown plants under controlled conditions to see how seeds produced under these conditions relate to the field conditions.
Chapter 3

Material and Methods

The field experiment was executed on a field at Wageningen University (51°59’05.7’’N, 5°39’29.2’’E, 25m above sea level), The Netherlands.

Plant materials and growth conditions

Field experiment

Arabidopsis genotypes (Landsberg erecta (Ler), Columbia (Col-0) and other genotypes with the Ler genetic background (five Near Isogenic Lines (NILs) - NILDOG1-Cvi (Cape Verde Islands), NILDOG2-Cvi, NILDOG3-Cvi, NILDOG6-Kas-2 (Kashmir), NILDOG22-An-1 (Antwerpen) (Alonso-Blanco et al., 2003; Bentsink et al., 2010), and the dog1-1 mutant (Bentsink et al., 2006) that we used in the experiments of Chapter 2 were subjected to the field conditions spanning all seasons of the year (spring, summer, autumn and winter). Plants were grown in spring (between April 2012 and July 2012), summer (between August 2012 and November 2012), autumn (between October 2012 and June 2013) and winter (between December 2012 and June 2013). They are respectively referred to as spring, summer, autumn and winter even though part of the life cycle occurred in another season.

Seeds were sown in trays, one row per genotype (Fig. 1A) followed by a 4-day cold treatment at 4°C, and transferred to a plastic tunnel without any environmental control to avoid possible damage by strong rain. Every two days the trays were watered for approximately two weeks until seedlings were fully established for spring, summer and autumn experiments. The winter experiment took 14 weeks because of the low temperatures that slowed down seed germination and subsequent seedling growth. Germinated seedlings were transferred to different trays (28 seedlings per genotype per tray with four replicates (trays) per genotype) (Fig. 1B). These trays were kept in the tunnel for one more week, to ensure the survival of seedlings (Fig.1C). After that, the trays were transferred to the field (Fig. 1D). Sensors to measure the soil temperature were evenly distributed over the trays and temperature data were recorded every 10 minutes. Data of air temperature was obtained from the weather station at De Bilt, The Netherlands, (http://www.knmi.nl/ klimatologie /daggegevens/selectie.cgi) and data of day length was obtained from (http://www.sunrise-and-sunset.com/nl/nederland/amsterdam).

Seeds were harvested when the plants had stopped flowering and more than 50% of the siliques were brownish. The seeds were harvested as a bulk from each tray.
Figure 1. Set up of the field experiment. A) Seeds were germinated in a tray; each row contains a different genotype. B) 28 seedlings per genotype were transferred to a single tray. C) The trays were kept in a tunnel for 1-2 weeks before transfer to the field. D) Overview of the field experiment.

Controlled conditions

We compared the results of the field conditions with the results of plants grown under controlled conditions in a growth chamber at 20°C/18°C (day/night) under 16h photoperiod of artificial light (µmol·m⁻²·s⁻¹) and 70% relative humidity, using the same genotypes as described above.

Germinated seedlings were grown on 4 x 4 cm Rockwool blocks. Plants were grown in three replicates, with five plants per replicate and were watered three times per week with a standard nutrient solution (Chapter 2, Table S1).

Seed performance analyses

Seed size was analysed by taking photographs of the seeds on white filter paper (20.2 x 14.3 cm white filter paper, Allpaper BV, Zevenaar, The Netherlands, http://www.allpaper.nl) using a Nikon D80 camera fixed to a repro stand with a 60mm
macro objective. The camera was connected to a computer with Nikon Camera Control Pro software version 2.0. Clustering of seeds was prevented as much as possible. The photographs were analysed using ImageJ (http://rsbweb.nih.gov/ij/) by combining colour thresholds (Y100-255U0-85V0-255) with particle analysis that automatically scored seed size as the area of selection in square millimeters.

Germination experiments were performed as described previously (Joosen et al., 2010). In brief, two layers of blue germination paper were equilibrated with 47ml demineralized water in plastic trays (15 x 21 cm). Six samples of approximately 50 to 150 seeds were spread on wetted papers using a mask to ensure accurate spacing. Piled up trays were wrapped in a closed transparent plastic bag. The experiment was carried out in a 22°C incubator under continuous light (143 µmol·m⁻²·s⁻¹). Pictures were taken twice a day for a period of 6 days using the same camera and software as described for seed size. Germination was scored using the Germinator package (Joosen et al., 2010). To measure the seed dormancy level (DSDS50; days of seed dry storage required to reach 50% germination) germination tests were performed weekly until all seed batches germinated for more than 90% (He et al., 2014).

Results

To investigate the influence of the seasons on seed dormancy, we performed a field experiment using eight different genotypes. These consisted of two wild types, Landsberg erecta (Ler) and Columbia (Col-0), and five Near Isogenic Lines (NILDOG1, NILDOG2, NILDOG3, NILDOG6, NILDOG22) that are known to be affected in seed dormancy (Bentsink et al., 2010), as well as a mutant with a lesion in the DOG1 gene (dog1-1).

Environmental conditions during the seasons

The conditions that the plant experienced in the different seasons we determined by monitoring the soil temperature (Fig. 2A), air temperature (Fig. 2B) and the day length (Fig. 2C). Both soil and air temperatures gradually increased during the spring, autumn and winter experiment, while they gradually decreased for plants of the summer experiment (Fig. 2A and B, respectively). During the spring experiment the day length increased from 14:17 hours to 16:44 hours per day, whereas in summer the day length decreased from 14:32 to 9:17 hours (Fig. 2C). The autumn and winter experiments, included the shortest day of the year, with only 7:43 hours of light. During these short days plants were in the rosette (autumn) and seedling (winter) stage. The reproductive period of these plants started when the day length increased again. Seeds were harvested at a day length of 16:38 hours for autumn and winter experiments (Fig. 2C).
Figure 2. The minimum, average and maximum temperature of the soil (A) and the air (B), and day length (C). From seed sowing to seed harvest of each experimental season (spring, summer, autumn and winter) is indicated by colour bars.
Effect of the seasons on the Arabidopsis life cycle

For every season the time between seed sowing and harvesting was recorded and the life cycle calculated. Plants that were sown during autumn and winter had the longest life cycles (264 and 183 days, respectively; Fig. 3). In both cases this was caused by the fact that these plants overwintered in the vegetative state and only started flowering in spring. This also indicates why seeds of both of these growths were harvested in June 2013. Spring and summer sown plants had shorter life cycles, with 71 and 99 days, respectively (Fig. 3). These values are comparable to the life cycles of the plants that were grown under controlled conditions, which took 72 days from sowing to seeds harvest.

Figure 3. Life cycle (days) of the plants grown in a controlled chamber (20°C/18°C (day/night) under 16h photoperiod of artificial light (150 µmol.m⁻².s⁻¹) and 70% relative humidity), and in different seasons (spring, summer, autumn and winter).

Influence of the seasons on seed performance

We assessed seed performance of plants that were grown in the field and compared this with performance of seeds produced under controlled conditions. Plants from seeds that were sown during the winter showed a higher number of branches, greater plant height and higher seed yield (data not shown). The others three seasons (spring, summer and autumn) displayed similar plant phenotypes.

Seed performance over the seasons varied. The smaller seeds (Fig. 4A) displayed the highest dormancy levels (higher DSDS50 values; longer after ripening (AR) time) (Fig. 4B). Seeds matured in the growth chamber produced the largest seeds with lower dormancy levels (Fig. 4).
Seed dormancy during the seasons

**Figure 4.** A) Seed size (mm$^2$) and B) Dormancy level (DSDS50) of plants grown in controlled conditions (chamber) and in different seasons (spring, summer, autumn and winter). Averages of four replicates are displayed. Error bars represent standard error. Means followed by the same letter did not differ by Tukey’s test (P<0.05).

**Genotype by environment interaction**

Dormancy levels and seed size were affected by both the environment and the genotype. All genotypes produced larger seeds in the growth chamber than in the field (Fig. 5). NILDOG2, NILDOG22 and NILDOG3 produced the smallest seeds in the chamber (Fig. 5A), whereas in the field Ler, dog1-1, NILDOG22 and NILDOG3 were the genotypes with the smallest seeds (Fig. 5B and S1).

Spring, autumn and winter experiments produced seeds with identical dormancy levels (Fig. 4B) and the differences in dormancy in these seasons were determined by the genotype (Fig. S2). An exception was NILDOG2, that had slightly lower dormancy level in the spring experiment than in the winter and autumn experiments (Fig. S2).

The genotypes grown under controlled conditions had a different dormancy behaviour compared to the ones grown in the field (Fig. 6A and 6B, respectively). For all genotypes, plants grown in the growth chamber released dormancy earlier (lower DSDS50 values; shorter AR time) than plants grown in the field, independent of the season (Fig. 6A and 6B).

The dog1-1 mutant is a non-dormant genotype and the seasons did not influence the very low DSDS50 values (Fig. S2). However, we could see an effect of the seasons when we investigated the after-ripening behaviour, since the maximum germination levels (Gmax) at different time points after seed harvest of seeds sown in spring and autumn were fully after-ripened (around 100% of germination) 6 days after harvest, whereas dog1-1 seeds sown in winter required 68 days to completely after-ripening and in summer even 111 days (Fig. 6C).
Figure 5. Seed size (mm$^2$): A) Seeds produced in controlled conditions, B) Average of all seasons for each genotype of seeds matured in the field. Averages of four replicates are displayed. Error bars represent standard error. Means followed by the same letter did not differ by Tukey’s test (P<0.05).

Discussion

Experiments that manipulate the environment during seed maturation are an important tool to understand the effect environmental factors to which the mother plant is exposed on subsequent seed performance (Donohue, 2009; He et al., 2014). By evaluating primary dormancy levels under laboratory conditions and in the field, Huang et al. (2010) found quantitative trait loci (QTL) for primary dormancy under controlled conditions that collocated with QTL for field germination phenology. This indicates that the genotypic effect remain in field conditions as well.

Seed performance in response to different seasons

Seeds are responsible for the development of the next generation, and how these seeds perform is determined by both the genotype and the environment of the mother plant (Elwell et al., 2011; He et al., 2014). For many species, adjusting the life cycle is a strategy to avoid sub-optimal environmental conditions and maximize survival and fitness of later stages of growth (Montesinos-Navarro et al., 2012). Seedling emergence is usually synchronized with seasonal changes in the environment (Fenner & Thompson, 2006; Baskin & Baskin, 2014). Earlier developmental stages, like seedlings are expected to be more sensitive to sub-optimal conditions than later stages, i.e. vegetative plants (Huang et al., 2010). However, it is a huge challenge to predict the effects of a changing environment on natural populations.
Seed dormancy during the seasons

Figure 6. A-B) Dormancy levels (DSDS50): A) Seeds produced in controlled conditions, B) Average of all seasons for each genotype of seeds matured in the field; C) Germination (%) of the dog1-1 mutant until it reached 100%. Averages of four replicates are displayed. Error bars represent standard error. Means followed by the same letter did not differ by Tukey’s test (P<0.05).

We have seen that life cycle strategies largely vary over the seasons. The life cycle of the plants that were sown during autumn and winter were the longest, with 264 and 183 days, respectively (Fig. 3), because these plants only started flowering in the spring. The delay of plant growth in these seasons is probably due to the low temperature, at which the risk of mortality or flower abortion is high (Masuda & Washitani, 1990). Plants that are sown in spring and summer have a comparable life cycle length (71 and 99 days respectively, Fig. 3). Life cycle strategies are normally determined by controlling the timing of germination and flowering (Donohue et al., 2005; Wilczek et al., 2009). In our experiment germination was controlled by us since we performed artificial stratification that allows a synchronized uniform germination of all genotypes. Furthermore the genetic material used did not vary much for flowering time except for the NILDOG2 lines, due to
the presence of the CRYPTOCHROME 2 (CRY2) Cvi allele. Under controlled conditions these lines flower slightly earlier than wild type Ler (El-Assal et al., 2001; Alonso-Blanco et al., 2003).

Plants from the spring experiment flowered, and produced seeds during summer and for plants sown in summer these events occurred during autumn, when temperature and day length started to decrease (Fig. 2). The photoperiod during seed maturation is a predictable indicator of the season (Munir et al., 2001). Postma & Ågren (2015) demonstrated differences in dormancy levels of seeds from two different field sites, one in Sweden and another in Italy. Conditions of the Swedish field resulted in more dormant seeds than those of the Italian field, and the difference may be related to the maternal photoperiod, since the average temperatures between the fields did not differ much. He et al. (2014) investigated seed performance in several different maternal environments and found that low light intensity and short days, as well as high temperature during seed maturation resulted in smaller seeds. In the current study we observed that plants sown during summer produced smaller seeds (Fig. 4A). These seeds developed when the days became shorter, and light intensities and temperature lower (Fig. 2).

Seeds matured during short days (summer experiment) produced seeds with deeper dormancy levels (Fig. 4B and S2). He et al. (2014) showed that photoperiod did not have any significant effect on seed dormancy, but low light intensity and low temperature enhanced the dormancy levels of Arabidopsis. Although Munir et al. (2001) suggested that the maternal photoperiod may contribute to variation in dormancy levels in Arabidopsis depending on progeny stratification. These authors showed that for seeds matured under short days, germination percentage and rate increased in stratified but were lower in unstratified seeds. Donohue et al. (2005) demonstrated that seeds matured under short days displayed slightly later germination than seeds matured under long days but the germination percentage was higher in seeds matured under short day conditions than seeds matured under long days.

By the end of the summer experiment the temperature started to decrease, especially during the phase of seed development (Fig. 2A and B) and the seeds were very dormant. It is known that low temperature promotes deep primary dormancy, whereas warm temperatures reduce dormancy (Schmuths et al., 2006; Chiang et al., 2011; Kendall & Penfield, 2012; Chiang et al., 2013; He et al., 2014; Huang et al., 2014). The induction of high dormancy at the end of summer might be important to avoid germination in autumn, and the cold experienced during the winter would ensure germination in the next spring when temperatures become favourable for germination (Probert, 2000; Bewley et al., 2013). According to Donohue (2009), germination is influenced by pre-dispersal seed maturation conditions and post-dispersal seasonal conditions. Therefore, both the influence of the environment during seed development and the link with the environmental conditions during seed germination are important for the establishment and depth of dormancy.
Montesinos-Navarro et al. (2012) simulated spring and autumn germination conditions. For autumn germination, seeds were kept moist with progressive reduction of temperature and hours of light (which is equivalent to our summer experiment). In the spring germination conditions seeds were kept dry and in the dark to simulate autumn, followed by 30 days at 4°C to simulate winter, then moistened and exposed to a progressive increase of temperatures and hours of day light (which is comparable to our winter experiment). These authors found that germination behaviour was significantly different in autumn versus spring simulated conditions, in which seeds exposed to autumn simulated conditions, displayed a decrease of 33% in final germination and a decrease in germination speed, as compared to spring germination conditions. Taken together these results confirm that temperature and day length are indeed important traits for dormancy/germination strategies in nature.

Genotypic response to the seasons

Most genotypes showed similar responses to the different seasons. For all of these, the summer experiment resulted in the highest dormancy level. However NIL DOG2 showed a strong interaction with the environment: in the spring experiment it displayed the lowest dormancy level, followed by winter, autumn and summer, respectively (Fig. S2). A strong interaction between natural variation and pre- and post-dispersal environment was found for recombinant inbred lines (RILs) population (Donohue et al., 2005). Genetic variation for maternal photoperiod and seasonal dormancy in RILs was also found by Munir et al. (2001). He et al. (2014) proposed that phenotypic plasticity varying among different genotypes is caused by genotype by environment interactions.

Strategies for breaking dormancy are genotype depend as well. In Col-0 dormancy can be broken by a short cold treatment, suggesting that the maturation of this genotype occurs in cool days of autumn and the seeds germinate in the following spring. However, Ler seeds need a warm period followed by a cold treatment in order to germinate, which suggests that the seeds mature in autumn, will not germinate in spring but only in the following autumn, because the seeds need to experience the warm temperatures of summer (Donohue et al., 2007). Also Schmuths et al. (2006) found variation in germination among natural accessions of Arabidopsis when seeds matured at low temperatures, but not in seeds that matured at high temperature. In a study of 18 wild accessions of Arabidopsis, differences in response to chilling (simulation of winter) for seeds maturated in different temperatures (20°C, 15°C and 10°C) was shown; some accessions did not respond to a cold treatment in the dark, independent of the temperature of maturation, although low temperatures during seed maturation were more effective to increase the dormancy levels in most of the accessions (Penfield & Springthorpe, 2012).
Field versus controlled conditions

The variations in temperature, rainfall, photoperiod and nutrients under field conditions normally hamper repeatability of experimental results. In our study, seed size and seed dormancy (DSDS50) from plants grown in a growth chamber differed from plants grown in the field. In the chamber, where the environment is controlled, plants produced larger seeds and these seeds were less dormant than seeds that were produced in the field, independent of the season (Fig. 4) or genotype (Fig. 5 and 6). A short exposure of plants to extreme environments such as heat, stress or drought during seed filling can decrease seed set, seed size, seed weight, reduce yield and also result in low seed quality (Singh et al., 2013). In the field such changes of the environment can occur suddenly, especially fluctuations in temperature.

To understand the differences in seed dormancy between seeds developed in the field and under controlled conditions (Fig. 4) we present three hypotheses. The first one is that in our settings the controlled growth chamber did not have fluctuations in temperature and photoperiod during the complete plant life cycle (20°C/18°C (day/night) under 16h photoperiod of artificial light (150 μmol·m⁻²·s⁻¹)), resulting in large seeds with low dormancy. This is opposite to what normally occurs in nature where fluctuations of the environment are prevalent during the life cycle of plants, which may cause deep dormancy as a survival mechanism against (short-term) changes in the environment. However, it is not known how alternating (diurnal) temperatures during plant growth influence seed dormancy. Only few studies have attempted to understand the physiological process by which alternating temperatures appear to be important for germination in dormant species, such as increased germination in the dormant ecotype Cvi of Arabidopsis (Ali-Rachedi et al., 2004) and various circadian clock mutants (Penfield & Hall, 2009). Indirect effects through the mother plant have been demonstrated by Kendall & Penfield (2012) in Arabidopsis, in which alternating the growth temperature of the mother plant, before and after flowering, influenced seed dormancy. Postma & Ågren (2015), studying three different maternal environments (one greenhouse environment and two field sites), found differences in dormancy levels among the environments. The greenhouse environments led to lower seed dormancy than the two field environments. This may be related to the temperature inside the greenhouse that was higher compared with the field sites.

The second hypothesis assumes that the combination of different environments causes differences. It is known that single environments, as described for temperature and photoperiod, may influence seed performance, especially seed dormancy, but a combination of environments may show a different response. He et al. (2014) reported that a combination of phosphate and temperature did not have a significant effect on plant and seed performance, while the individual environments, phosphate or temperature, did show an effect.
The third hypothesis to explain the difference in seed dormancy of plants grown under controlled conditions and in the field (Fig. 4B), assumes the existence of nutrient effects on dormancy. Under controlled conditions, nutrients were provided to the mother plant every two days as a standard nutrient solution (Chapter 2, Table S1) whereas in the field experiment no additional nutrients were supplied to the plants. The nutrient composition and levels of the soil were not analysed. The addition of nutrient fertilizer to parental plants decreases dormancy in the seeds of several species (Fenner, 1991). However, Arnold et al. (1995) observed that reduced potassium nutrition in developing seeds of Sorghum bicolor increased the germinability, because the ABA content of the seeds was reduced. Conditions favoring nitrate accumulation in mother plants of Arabidopsis resulted in lower dormancy (Alboresi et al., 2005; He et al., 2014). Matakiadis et al. (2009) reported that high nitrate concentrations may release seed dormancy in Arabidopsis, in part by reducing abscisic acid levels. A similar effect was found by Modi & Cairns (1994) who observed that molybdenum deficiency in wheat resulted in lower seed dormancy by decreasing abscisic acid content. It is likely that a deficiency of molybdenum, a co-factor of nitrate reductase, reduces nitrate reductase activity, resulting in higher nitrate levels of the seeds. Phosphate levels did not influence dormancy, but increasing phosphate and phytate content increased germination under stress conditions (He et al., 2014).

**Conclusions**

Our study indicates that, depending on the time of the year when plants are grown, seed performance can be different. Plants grown during summer, when the days began to become shorter and the temperature started to decrease, produced smaller seeds with deeper dormancy. Seed dormancy was largely controlled by the environment seen the fact that the differences caused by the different seasons are larger than the differences among the genotypes. In addition, plants grown in a controlled environment produced larger seeds with lower dormancy than those grown in the field.

**Supplementary Material**

Supplemental files can be downloaded from:
http://www.wageningenseedlab.nl/thesis/dsvidigal

**Figure S1.** Seed size per genotype in different seasons (Spring, Summer, Autumn and Winter).

**Figure S2.** Dormancy level (DSDS50) per genotype in different seasons (Spring, Summer, Autumn and Winter).
References


Chapter 4

Altitudinal and climatic adaptation of seed dormancy-related traits in Iberian *Arabidopsis thaliana*

Abstract

The question how plants respond and adapt to different environmental conditions has attracted the attention of plant researchers from different areas, such as ecology, genetics and physiology. In order to identify environmental factors (climatic, edaphic and ecological factors) that might drive the evolution of seed dormancy-related traits in *Arabidopsis*, a regional collection of 300 wild *Arabidopsis* accessions collected from different locations in the Iberian Peninsula, was used. Spatial autocorrelation patterns of phenotypic traits and environmental variables showed that seed dormancy, rate of germination and flowering time are significantly and positively spatially autocorrelated. Dormancy, seed size and flowering time showed strong altitudinal clines, indicating that *Arabidopsis* accessions from high altitudes flower later and produce larger seeds with lower dormancy. Seed dormancy, flowering time and seed size displayed the highest significant correlation coefficients with the environmental factors, in particular with average annual temperature. Summer precipitation also correlated significantly with seed dormancy, rate of germination and seed size. Overall, the high correlations of phenotypic traits and climatic parameters confirmed that dormancy, seed size and flowering time are relevant adaptive traits. Finally, early flowering and higher dormancy are likely mediated by *delay of germination 1 (DOG1)* expression as part of a mechanism to adapt to minimum temperature and humanised habitat.
Introduction

*Arabidopsis thaliana* (Arabidopsis) is an annual plant native to Europe and Middle Asia that occurs across a broad latitudinal, longitudinal and altitudinal range spanning highly diverse habitats (Hoffmann, 2002). This extensive distribution range in the northern hemisphere contains an enormous intraspecific phenotypic and genotypic variation, which is presumed to be involved in adaptation to contrasting environments (Atwell *et al.*, 2010; Hancock *et al.*, 2011; Lasky *et al.*, 2012). In particular, this geographic distribution includes substantial variation for the life cycle of natural populations of Arabidopsis, which have been classified as winter and spring annuals (Donohue, 2002; Picó, 2012; Montesinos-Navarro *et al.*, 2012). Adapted life cycles are an important survival strategy of plants to avoid harsh environments by reproducing only when environmental conditions are favourable for seedling establishment (Donohue *et al.*, 2005a; Donohue *et al.*, 2005b). Plants with the spring life cycle germinate, grow to maturity, bloom, set seed and disperse their seeds in the same spring or summer season. By contrast, plants with winter life cycles, germinate in autumn, overwinter as seedlings or rosettes, and flower and disperse seeds in next spring (Donohue, 2002). The overall life cycle of annual plants like Arabidopsis is largely determined by the timing of germination under natural conditions, which is controlled by the level of seed dormancy and by the timing of flower initiation. Both traits show substantial natural variation among wild accessions of Arabidopsis (Lempe *et al.*, 2005; Kronholm *et al.*, 2012), but they are also influenced by the environmental maternal conditions (Donohue *et al.*, 2005a; Manzano-Piedras *et al.*, 2014; He *et al.*, 2014).

Seed dormancy contributes to the adaptation of plants to their environment by optimizing the germination to the right period of the year (Donohue *et al.*, 2005b). It prevents germination even though conditions like water, light, temperature, and nutrients are suitable for germination (Fenner & Thompson, 2006). Multiple environmental factors during seed maturation can affect dormancy in Arabidopsis. It has been shown that low nitrate concentrations (Alboresi *et al.*, 2005; Matakiadis *et al.*, 2009; He *et al.*, 2014), low temperatures (Donohue *et al.*, 2008; Dechaine *et al.*, 2009; Kendall & Penfield, 2012; He *et al.*, 2014; Huang *et al.*, 2014), low light intensity (He *et al.*, 2014) and long-day photoperiod (Munir *et al.*, 2001; Postma & Ågren, 2015) may increase seed dormancy. Thus, seed dormancy appears as a complex trait that has been acquired during the evolution of many species as a physiological mechanism for plant adaptation to the environment (Bewley *et al.*, 2013). This is supported by the geographic distribution of plant species differing in their overall seed dormancy, since the number of species that exhibit high dormancy increases with latitude (Bewley *et al.*, 2013). In addition, the large amount of intraspecific variation present in Arabidopsis has been associated with the latitude of origin of populations, because wild accessions from northern Europe display, on average, lower dormancy levels than accessions from southern Europe (Chiang *et al.*, 2011; Debieu *et al.*, 2011).
Seed dormancy of Iberian Arabidopsis

Accordingly, seed dormancy is affected by environmental and genetic factors (Bentsink & Koornneef, 2011), and genotype by environment interactions reflect the genetic variation for the phenotypic plasticity of this trait across different environments (Donohue, 2009; Kendall & Penfield, 2012; He et al., 2014; Footitt et al., 2014).

Over the past decade, the genetic architecture of the natural intraspecific variation for seed dormancy of Arabidopsis has been dissected by quantitative trait locus (QTL) analyses in several experimental populations (van Der Schaar et al., 1997; Alonso-Blanco et al., 2003; Clerkx et al., 2004; Meng et al., 2008; Laserna et al., 2008; Bentsink et al., 2010). Delay of Germination 1 (DOG1), which was first identified in the Landsberg erecta (Ler) and Cape Verde Islands (Cvi) Recombinant Inbred Line (RIL) population (Alonso-Blanco et al., 2003), appeared as the main dormancy QTL contributing to Arabidopsis natural variation. DOG1 was cloned by map-based cloning and was shown to encode a protein of unknown function (Bentsink et al., 2006). The dog1 mutant shows no dormancy after harvest, indicating that DOG1 is a key player essential for the induction of seed dormancy. In addition, the amount of DOG1 protein has been correlated with the depth of dormancy at the end of seed maturation (Nakabayashi et al., 2012). The relevance of DOG1 gene has been further confirmed by its identification in other Arabidopsis populations (Bentsink et al., 2010; Kendall et al., 2011; Kronholm et al., 2012; Postma & Ågren, 2015), as well as in some other species (Graeber et al., 2010; Ashikawa et al., 2014; Guo et al., 2015; Née et al., 2015).

DOG1 expression is seed specific and peaks during the last phases of seed development (Bentsink et al., 2006). DOG1 controlled dormancy is largely mediated by differences in DOG1 expression as was shown by the analysis of different natural variants (Bentsink et al., 2006) as well as by the effect of the maternal temperature on DOG1 expression and dormancy (Chiang et al., 2013). DOG1 expression is also associated with geographical variation, with southern accessions having DOG1 expression earlier during seed development and at higher levels than northern accessions (Chiang et al., 2011). Moreover, also structural changes in DOG1 appear to affect seed dormancy and, using $F_{ST}$ /$Q_{ST}$ comparisons, which the differentiation among populations is estimated for a set of molecular markers ($F_{ST}$) and it is compared to the same measure of differentiation at a set of quantitative traits ($Q_{ST}$), Kronholm et al. (2012) suggested that DOG1 contributes to local adaptation.

Despite current progress in understanding the genetic mechanisms of seed dormancy and its natural genetic variation in Arabidopsis, the environmental factors that contribute to maintaining this natural variation remain largely unknown. Common garden or reciprocal transplant experiments are robust approaches used to understand the association of the environment and evolutionary history (genotype and/or genotype by environment interactions) to phenotypic traits (Anderson et al., 2014). Geographic and environmental clines have often been detected for important adaptive traits such as flowering time (Stinchcombe et al., 2004; Caicedo et al., 2004; Hancock et al., 2011).
However, these analyses require the selection of an appropriate set of populations spanning the range of variation of the relevant environmental factors, as well as their environmental documentation. In addition, such genetic-environmental correlation analyses require geographically-explicit approaches to consider spatial autocorrelation patterns affecting the independence among samples (Sokal & Oden, 1978).

Recently, several new collections of wild accessions have been developed from different world regions (Le Corre, 2005; Samis et al., 2012; Brachi et al., 2013; Long et al., 2013). The use of world-wide collections is hampered by low frequency alleles (Cao et al., 2011), while regional collections are expected to have a higher frequency of the alleles accounting for the phenotypic variation. An example of such a regional collection is the set of wild Arabidopsis accessions that were collected in the Iberian Peninsula (Picó et al., 2008; Méndez-Vigo et al., 2011; Manzano-Piedras et al., 2014). This regional collection provides an ideal scenario to evaluate Arabidopsis climatic adaptation because the Iberian Peninsula is part of the species native range (Hoffmann, 2002), spans a large diversity of climates, altitudes (0-2600 m) and ecological habitats (Myers et al., 2000; Ninyerola et al., 2000; Manzano-Piedras et al., 2014), and has been shown to contain the largest amount of genetic variation of Arabidopsis in Eurasia (Picó et al., 2008; Cao et al., 2011). These accessions are being used to study the evolutionary effects of the environment on natural genetic variation for flowering time (Méndez-Vigo et al., 2011; Manzano-Piedras et al., 2014). In addition, analysis of a smaller set of populations from this collection has shown that the Iberian Peninsula contains as much diversity for seed dormancy as accessions from the rest of the world (Kronholm et al., 2012).

In order to identify environmental factors that might drive the evolution of seed dormancy in Arabidopsis, we have studied this regional collection of 300 accessions from the Iberian Peninsula, in relation to climatic, edaphic and ecological factors, by analysing seed dormancy and other life history traits, such as the rate of seed germination, seed size and flowering time. In addition, DOG1 expression levels were analysed in a subset of the population to reveal whether DOG1 could be the molecular basis for the detected climatic and environmental adaptation.

Materials and Methods

Plant material and growth conditions

A regional collection of 300 wild accessions of Arabidopsis (Arabidopsis thaliana) collected from different local populations from the Iberian Peninsula was analysed (Picó et al., 2008; Méndez-Vigo et al., 2011; Manzano-Piedras et al., 2014). To obtain the samples of seeds for the analyses of seed traits, all accession were multiplied in a single experiment containing 6 replicates per accession and one plant per replicate in a greenhouse at Wageningen University (51°59’05.7”N, 5°39’29.2”E, 25m above sea level),
The Netherlands, in 2013. To synchronize the seed production of all accessions, flowering initiation was accelerated by a vernalization treatment. However, since accessions differ in their vernalization response (Méndez-Vigo et al., 2011), the collection was planted on three different dates and received three different vernalization treatments to ensure synchronous flowering initiation and seed production. Accessions were classified as late (168 accessions), intermediate (98 accessions) and early (34 accessions), and received 8, 4 or 2 weeks of cold treatment, respectively.

For multiplication of the collection, seeds were sown in Petri dishes on water soaked filter paper and incubated for four days in a cold room at 4 °C in the dark to break dormancy (seed stratification). Subsequently, the Petri dishes were transferred to a germination cabinet at 22 °C (16 hours light per day) for four days before planting. Germinated seedlings were transferred to a greenhouse on 4 × 4 cm² rockwool plugs and watered with 1 g/l Hyponex fertilizer (NPK = 7:6:19). After 3 weeks, the plants were moved to a climate chamber for vernalization (4°C; 70% RH; 12 h of light). Three different times of vernalization treatment were given, as described above and shown in Table S1. Subsequently, the plants were moved again to the greenhouse and grown in a complete randomized block design with 6 replicates. Six or five replicates were harvested form 252 accessions, three or four replicates could be harvested for the remaining accessions (Table S2), but only three accessions had two replicates and one accession with one replicate. Due to the high correlation among replicates for all of the population, we decided to include these four accessions that presented less than three replicates in forward analysis.

**Seed germination and dormancy determinations**

To measure seed dormancy, germination tests were performed weekly until dormancy had been released from all accessions (> 90% of germination). Germination experiments were performed in plastic (15 × 21 cm²) trays containing 47 ml water and two layers of blue filter paper. Six samples of approximately 50–100 Arabidopsis seeds were dispersed on the filter paper, using a mask to ensure accurate and reproducible spacing. Clustering of seeds was prevented as much as possible. Trays were kept in an incubator at 22 °C and constant light, during five days. Photographs were taken once a day and they were analysed by the Germinator package (Joosen et al., 2010) to calculate the maximum percentage of germination (Gmax). Seed dormancy was quantified as DSDS50 (days of seed dry storage required to reach 50% of germination), which was calculated according to He et al. (2014). In addition, seed dormancy was also estimated as DSDS10 and DSDS90 (days of seed dry storage required to reach 10% and 90% of germination, respectively) values calculated from germination-time fitted curves, as the closest time point to obtain 10 or 90 percent of germination, respectively.

Germination after cold stratification (GAS) was estimated 55 days after harvest (DAH). For that, imbibed seeds were placed for 10 days at 4°C and thereafter they were
transferred to an incubator at 22°C and constant light. Photographs were taken three times a day and maximum germination percentage was estimated as described above. In addition, these pictures were used to measure the rate of germination with three different variables that were calculated with the Germinator package: 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).

**Seed size analysis**

Seed size was analysed by image analysis from photographs of the imbibed seeds on blue filter paper 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. Clustering of seeds was prevented as much as possible. Photographs were analysed using ImageJ (http://rsbweb.nih.gov/ij/) by combining colour thresholds \(Y_{0-255}U_{0-255}V_{140-255}\) with particle analysis.

**Flowering time**

Flowering time (FT) was measured as the number of days from the planting date until the appearance of the first flower. For this, plants were grown at the CNB-CSIC, Spain, in a growth chamber at 21°C and with a long-day photoperiod (16 hours light; 8 hours darkness), as previously described by Méndez-Vigo et al. (2011). All accessions were grown simultaneously in a single experiment organized in a two-complete-blocks design, and including six plants per accession in each pot and block. The experiment was finished after 220 days, and this FT value was given to accessions that had not flowered at that time. These non-flowering accessions correspond to about 20% of genotypes from the Iberian Peninsula that have been previously shown to have an obligate vernalization requirement (Méndez-Vigo et al., 2011).

**Geographical and environmental data**

A total of 300 wild local populations of Arabidopsis were surveyed in a region of around 800 x 700 km\(^2\) of the Iberian Peninsula (Fig. 1). Populations were *in situ* georeferenced for their latitude, longitude, and altitude with a global positioning system receiver (Table S2). They were spaced at an average distance of 357 ± 202 km, with a minimum and maximum of 1 and 1042 km respectively. Altitudes ranged from 0 to 2200 m above sea level. Seeds from a single random individual per accession were collected and analysed in this study. The collection of 300 accessions is available through the Nottingham
Arabidopsis Stock Centre (http://arabidopsis.info). Environmental information (including climatic landscape use and soil pH) have been described previously by Méndez-Vigo et al. (2011) and Manzano-Piedras et al. (2014). Climatic data of each population location were obtained from the Digital Climatic Atlas of the Iberian Peninsula (http://www.opengis.uab.es/wms/iberia/index.htm) which was developed at a 200-m resolution following the climatic models described by Ninyerola et al. (2000). Models were based on meteorological records of 15 to 50 years, for the period 1950 to 1999, from 2285 meteorological stations located across the Iberian Peninsula. Climatic variables were obtained for each location: mean monthly and mean annual temperature, mean minimum and maximum monthly and annual temperature, total monthly and total annual precipitation, and mean monthly and mean annual solar radiation, as well as the population habitats that were quantified as the proportions of anthropic and natural types of vegetation cover in each location, which were estimated from the CORINE Land Cover Map (http://www.iede.es). The land cover in a 78-ha circular area around the global positioning system coordinates of each location was classified as the proportion of the following categories: urban, crops, bushes, and woods. Anthropic and natural land cover was estimated by summing the proportional cover of urban and crops, and bushes and woods, respectively. Finally, soil pH was obtained from the Soil Geographical Database of Eurasia v.4 (http://eusoils.jrc.ec.europa.eu).

**DOG1 expression**

To analyse *DOG1* gene expression, 100 accessions of the Iberian collection covering the whole dormancy range were selected for expression analyses. Three biological replicates of 100 selected accessions were used for *DOG1* RT-qPCR.

**RNA isolation:** RNA was isolated using the Nucleospin RNA plant kit (Macherey-Nagel: 740949) according to the manufacturer’s protocol with some 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 (Biok: TR230300) and measured on an Xpose (Biok: TR112003). RNA integrity was checked on a 1% agarose gel.

**cDNA synthesis and RT-qPCR:** 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) was added and supplemented with water to 10µL. The RT-qPCR was performed on a CFX connect (Bio-Rad).
To analyse DOG1 expression, two sets of primer pairs were designed in regions that were shown to be conserved among 19 accessions of the Iberian population, to avoid that possible mutations of the gene would interfere with the results (Fig. S1): Primer pair 1 (intron/exon border: FW: CGGCTACGAATCTTCCAGGTGG; REV: CGTTAGGCTCTCCGACATT; Primer pair 2 (over intron): FW: TTGGATGGGTGGTTGTCG; REV: CTCCGCCTTAAAGTCGCT. DOG1 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). Due to the large number of samples, several qPCR plates had to be used and to correct for this 3 replicates of pooled RNA of all the samples were included on each plate, using the primers of reference gene At4g12590. (Hellemans et al., 2007).

**Data analysis**

Correlation analysis among seed dormancy-related traits (i.e. seed dormancy, flowering time, rate of germination and seed size) was estimated with Dutilleul’s modified t test, which corrects the variance of the test statistically and the degrees of freedom according to the extent of spatial autocorrelation of each variable (Dutilleul, 1993; Méndez-Vigo et al., 2011; Manzano-Piedras et al., 2014). The relationship between seed dormancy-related traits and environmental variables (i.e. altitude, climate, land use and pH) were tested with simultaneous autoregressive models (SAR), which is a regression technique, based on generalised least squares (GLS), that estimate regression parameters taking spatial patterns of data into account by including the autocorrelation matrix of the errors (Beale et al., 2010). Both Dutilleul’s t-test and SAR were conducted using the software SAM (Rangel et al., 2010). Also, spatial autocorrelation patterns of environmental variables and seed dormancy-related traits were analysed according to Méndez-Vigo et al. (2011) and Manzano-Piedras et al. (2014), using the software PASSaGE v.2 (Rosenberg & Anderson, 2011). For each environmental variable and seed dormancy-related traits, Moran’s I autocorrelation coefficients were computed (Moran, 1950) and their significance was estimated from 1000 permutations. Finally, to check the robustness of patterns obtained with SAR models, a canonical correlation analysis (CCA) was conducted using SYSTAT v.13 using a set of selected environmental variables and the set of phenotypic traits according to Manzano-Piedras et al. (2014).
Table 1. Dutilleul’s correlation between seed dormancy-related traits of 300 Arabidopsis accessions *.

<table>
<thead>
<tr>
<th></th>
<th>DSDS 10</th>
<th>DSDS 50</th>
<th>DSDS 90</th>
<th>GAS</th>
<th>t10</th>
<th>t50</th>
<th>U8416</th>
<th>SS</th>
<th>FT</th>
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<tr>
<td>DSDS 10</td>
<td></td>
<td></td>
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<tr>
<td>DSDS 50</td>
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<tr>
<td>DSDS 90</td>
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<td>GAS</td>
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<td>t10</td>
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<td>0.901</td>
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<td>-0.097</td>
<td>0.001</td>
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<tr>
<td>FT</td>
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<td>-0.219</td>
<td>-0.180</td>
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* DSDS10, days of seeds dry storage required to reach 10% of germination; DSDS50, days of seeds dry storage required to reach 50% of germination; DSDS90, days of seeds dry storage required to reach 90% of germination; GAS, germination after stratification; t10, time required for 10% of viable seeds to germinate; t50, time required for 50% of viable seeds to germinate; U8416, uniformity of germination is the time interval between 84% and 16% of viable seeds to germinate; SS, seed size; FT, flowering time; DOG1 expression.

Significance: **, $P < 0.0006$; *, $P < 0.05$; ns, non-significant.

Results

Natural variation for seed dormancy-related traits in the Iberian Peninsula

The Iberian Peninsula collection of 300 natural Arabidopsis accessions (Fig. 1) was grown under the same conditions, in growth chamber at the CNB-CSIC in Spain, and flowering time (FT) was calculated. In an independent experiment, the Arabidopsis collection was grown in greenhouse at Wageningen University, the Netherlands. The harvested seeds were analysed for seed dormancy levels (DSDS10, DSDS50 and DSDS90), germination percentage after stratification (GAS), rate of germination (t10, t50 and U8416).
after stratification and seed size (SS). At 40 days after harvest (DAH) only 39 accessions (13%) germinated in a germination assay and the average germination of these accessions was less than 25% (data not shown). To test if this low germination was due to high levels of seed dormancy or to low seed viability, seeds were stratified for 10 days at 4°C at 55 DAH. After this treatment, seeds were tested for germination and GAS, t10, t50 and U8416 were calculated. Most accessions of the collection (74%) now germinated to over 90% (Fig. 2) indicating that their seeds respond strongly to stratification and that dormancy was broken by the cold treatment. However, 30 accessions showed GAS values lower than 50%, which indicates that 10% of the Iberian accessions respond only slightly to low temperature stratification (Table S2). Dormancy release of the collection was followed by monthly germination tests for nearly two years (559 days). From these data, seed dormancy levels, quantified as DSDS10, DSDS50 and DSDS90, were calculated. Correlation analyses among the different seed germination traits showed strong correlation between t10 and t50, as well as among DSDS10, DSDS50 and DSDS90 (Table 1). Therefore only DSDS50, GAS, t50, U8416, SS and FT were analysed further.

Figure 1. Geographical distribution of Arabidopsis accessions in the Iberian Peninsula. Dormancy expressed as DSDS50 (days of seed dry storage required to reach 50% germination) are indicated in different colours: white (0-100 days); grey (100-200 days); black (200-300 days); green (300-400 days); red (400-500 days) and blue (> 500 days).
As shown in Figure 2, all traits showed substantial genetic variation among the accessions. Overall, the traits t50, U8416 and SS showed normal distribution patterns, with a two to five-fold variation (Fig. 2). The distribution of GAS was skewed towards 100%, since most of the lines germinated fully after the stratification treatment (Fig. 2). In contrast, DSDS50 showed a tri-modal frequency distribution, with peaks of accessions corresponding to low, intermediate and high dormancy around values of 100, 300 and 550 days. In addition, FT showed a bi-modal distribution (Fig. 2), with a group of accessions flowering around 70 days after planting and another group that did not flower until the end of the experiment. The latter flowering corresponds to the 21% of Iberian accessions that have an obligate vernalization requirement (quantified as 220 days), which is in agreement with previous observations (Méndez-Vigo et al., 2011).

To determine the genetic relationship among the different types of traits, we analysed their correlation (Table 1). DSDS50 showed positive correlation with t50 and strong and negative correlation with SS and FT (Table 1). In addition, FT also showed negative correlation with t50 and positive correlation with SS. Furthermore, GAS showed negative correlation with the rate of germination traits and SS, whereas t50 had strong and positive correlation with U8416 (Table 1). Together, these results indicate that under our laboratory conditions, Arabidopsis accessions that flower earlier produce smaller seeds, which are also more dormant, and germinate more slowly after cold treatment.

Figure 2. Frequency distributions of seed dormancy-related traits in the Iberian Arabidopsis collection. A) Seed dormancy (DSDS50: days of seeds dry storage required to reach 50% of germination); B) Germination after stratification; C) t50 (time required for 50% of viable seeds to germinate); D) U8416 (uniformity of germination is the time interval between 84% and 16% of viable seeds to germinate); E) Seed size; F) Flowering time.
Geographical distribution of seed dormancy-related traits.

To test if the natural variation for the seed dormancy-related traits may be involved in adaptation to different environments we first analysed the spatial autocorrelation pattern of the traits by means of Moran’s $I$ test. DSDS50 (Moran’s $I = 0.19$), $t_{50}$ (Moran’s $I = 0.13$) and FT (Moran’s $I = 0.18$) showed a significant ($P < 0.05$ in all cases) spatial autocorrelation, which means that geographic and phenotypic distances were correlated, indicating that populations that are located geographically closer, are genetically more similar for these traits. However, the remaining traits showed weak or non-significant spatial autocorrelations, suggesting that their genetic variation shows a rather random spatial distribution. The largest geographical distance between accession pairs with significant autocorrelation was about 210 km for DSDS50, 180 km for $t_{50}$ and 280 km for FT. Therefore, these traits are not randomly distributed across the Iberian geography, suggesting that the variation for them might be shaped by environmental factors showing similar patterns of spatial autocorrelation, such as climatic parameters.

Since this collection of accessions is distributed across an altitudinal range of more than 2000 m (Fig. 1), we next analysed their altitudinal distribution as a geographical proxy for climatic variation. No clinal variation was detected for the rate of germination (Fig. S2). However, the variation for DSDS50, SS and FT displayed strong altitudinal clines, in such a way that the higher the altitude, the larger the seeds, the lower the seed dormancy level and the later the flowering time (Fig. 3A and B). These altitudinal clines accounted for 38.0, 21.2 and 37.8% of the phenotypic variance for DSDS50, SS and FT, respectively. Interestingly, nearly all accessions with extremely deep dormancy (DSDS50 > 400) and extremely early flowering (FT < 50 days) appeared to be distributed below 1000 m (Fig. 3C and 3E, respectively), indicating that populations with high seed dormancy and early flowering are not maintained in this region at high altitude. By contrast, accessions with very low seed dormancy, or very late flowering time, are found along the complete altitudinal range, supporting that the life cycles determined by these behaviours are adapted to a wider environmental range.
Figure 3. (A-B) Altitudinal clines of seed dormancy-related traits in Arabidopsis: A) Seed dormancy (DSDS50: days of seeds dry storage required to reach 50% of germination) and seed size (SS) at different altitudes; B) Flowering time (days) at different altitudes. (C-F) Altitudinal and climatic distributions for seed dormancy and flowering time: C) seed dormancy distribution over altitudes; D) seed dormancy distribution over mean annual temperature; E) flowering time distribution over altitudes; F) flowering time distribution over mean annual temperature. In A and B, data points are means ± SE accessions of accessions classified six altitudinal ranges given in meters above sea level.
Environmental distribution of seed dormancy-related traits.

In order to dissect the geographical patterns into environmental clines we first analysed the correlation between seed dormancy-related traits and various environmental factors, including climatic factors, the anthropic or natural habitat of the populations and the pH of the soil. DSDS50 and GAS correlated positively with the percentage of humanised habitat and negatively with pH of the soil. In contrast, SS and FT were negatively correlated with habitat and positively correlated with the pH. Analyses of the mean annual values of climatic variables detected significant climatic clines for DSDS50, SS and FT, thus accounting for 45.9, 21.3 and 36.8% of the phenotypic variance, respectively (Table S3). The strongest clines were found with the mean annual temperature, which appeared to be correlated positively with DSDS50 ($r = 0.538; P < 0.0006$) and $t50$ ($r = 0.218; P = 0.003$) and negatively with SS ($r = -0.446; P < 0.0006$) and FT ($r = -0.555; P < 0.0006$). Overall, accessions from local populations exposed to warmer mean annual temperature flowered earlier and produced smaller and more dormant seeds (Fig. 3D and 3F, Table S3). All the extreme dormant and very early flowering accessions came from populations with mean annual temperature higher than 11°C or 8.8 °C, respectively (Fig. 3D and 3F). In agreement with the observed altitudinal clines, accessions with very low seed dormancy or very late flowering time span the complete range of variation for mean annual temperatures (4.9°C to 15.5°C) (Fig. 3D and 3F).

To analyse in detail the relationship between seed dormancy-related traits and climate we applied simultaneous autoregressive models (SAR) to monthly climatic variables over the year (Fig. 4, Table S3). Seed dormancy showed significant correlations over the year with all climatic parameters. In particular, DSDS50 showed a strong positive correlation with minimum and maximum temperature over the year, but negative correlation with precipitation in spring and summer seasons (Fig. 4, Table S3). In addition, DSDS50 displayed a weak positive correlation with the potential solar radiation from April to September. By contrast, GAS only correlated weakly with precipitation during August and September (Table S3). The rate of germination, measured as $t50$, showed similar but weaker correlation than seed dormancy for maximum and minimum temperature, as well as for precipitation along the year (Fig. 4, Table S3). However, as expected from the negative correlation between SS and DSDS50 mentioned above, SS showed similar climatic correlation patterns over the year, but with opposite sign, for temperature, precipitation and solar radiation (Fig. 4, Table S3). Furthermore, FT correlated negatively with minimum and maximum temperature, as well as with fall and winter precipitation, whereas it showed positive correlation with summer precipitation (Fig. 4, Table S3). DSDS50, GAS, SS and FT also showed associations with humanised habitat (Table S3).
Figure 4. Relationship between seed dormancy-related traits and monthly climatic variables throughout the year. Each panel shows the correlation coefficients between the phenotypic traits indicated and monthly minimum temperature (○), maximum temperature (□), precipitation (□) and potential solar radiation (◇). Months on the x-axis are indicated with the first letter of the month. Yellow colours indicate $P<0.006$, blue colours indicate $P<0.05$ and no colour indicates non-significant coefficients tested by SAR models. DSDS50: days of seeds dry storage required to reach 50% of germination; SS: seed size; t50: time required for 50% of viable seeds to germinate; and FT: flowering time.

To test further the robustness of environmental patterns obtained with SAR models, we also conducted a complementary approach by performing canonical correlation analyses (CCA), which includes simultaneously multiple environmental variables and phenotypic traits. Since climatic variables showed strong correlation among them (Table S4), we only included the 11 environmental variables displaying correlation coefficients lower than 0.75. CCA generated four significant canonical correlation variates (Table 2), although the first variate was the most important since it was almost two-fold higher than the others ($1^{st}$ coefficient = 0.78; $2^{nd}$ coefficient = 0.43; $3^{rd}$ coefficient = 0.34 and $4^{th}$ coefficient = 0.30). These analysis showed clearly that BIO1 (mean annual temperature) exhibited a negative correlation with seed dormancy (-0.78) and a positive correlation with
flowering time (0.87) (Table 2). Thus, higher temperatures correlate with higher seed dormancy and earlier flowering. The second canonical variate showed a negative correlation between the rate of germination ($t_{50}$ and U8416), temperature seasonality (BIO4) and precipitation seasonality (BIO15) (Table 2). These phenotypic traits correlated also positively with annual precipitation (BIO12) and precipitation in June (RAIN6) (Table 2), indicating that accessions that come from drier places tend to germinate faster than those from wetter places. For the sake of clarity, we do not interpret the other two variates as they showed lower canonical correlation coefficients (Table 2) and the combination of phenotypic traits and environmental variables are not easy to explain. Overall, these results highlight the fact that temperature, seed dormancy and flowering time are all related.

**Association between DOG1 expression and seed dormancy-related traits**

$DOG1$ is the main seed dormancy gene that is related to natural variation so far. To determine if $DOG1$ gene expression is involved in the earlier detected environmental and climatic adaptation, 100 accessions of the Iberian collection covering the whole dormancy range were selected for expression analyses. The distribution of $DOG1$ expression showed a uni-modal distribution with a peak between 0.1 to 2.5 (Fig. 5A). Two accessions showed very high $DOG1$ expression levels (over 16-fold higher than the reference gene) and had a DSDS50 of 559 and 377, respectively. $DOG1$ expression positively correlated with DSDS50 and negatively with FT (Table 1 and Table S5).

$DOG1$ expression did not show any autocorrelation pattern, which means that geographical distance has little to do with $DOG1$ expression variation. In general, $DOG1$ expression correlated weakly with environmental variation. $DOG1$ displayed a positive correlation with minimum temperature from February to April (Fig. 5B and Table S3), with mean minimum annual temperature and with mean annual temperature (Table S3). Interestingly $DOG1$ expression variation seemed to be also associated with humanised habitat (Table S3).
Table 2. Canonical correlations between phenotypic traits and environmental variables of 300 Arabidopsis accessions from the Iberian Peninsula *.

<table>
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<tr>
<th>Group</th>
<th>Variables</th>
<th>Variate correlation*</th>
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<td>Phenotypic traits</td>
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* Results for the first four significant canonical variates are given. Only variables with correlation coefficients lower than 0.75 (positive or negative) were used for the analysis. Canonical correlations of seed dormancy-related traits and environmental variables above the overall canonical correlation for each canonical variate are highlighted. SS: seed size; GAS: germination after stratification; t50: time required to reach 50% germination of the total number of germinated seeds; U8416: uniformity of germination is the time interval between 84% and 16% of viable seeds to germinate; DSDS50: days of seeds dry storage required to reach 50% of germination; FT: flowering time; BIO1: annual mean temperature; BIO3: isothermality; BIO4: temperature seasonality; BIO8: precipitation of warmest quarter; BIO12: annual precipitation; BIO15: precipitation seasonality; RAIN6: precipitation in June; RAD6: potential solar radiation in June; OPEN: open spaces with little or no vegetation; HUMAN: sum of agricultural areas, artificial surfaces and open spaces with little or no vegetation; PH: soil pH.
Figure 5. A) Frequency distribution of DOG1 expression in a set of 100 accessions of the Iberian collection of Arabidopsis; B) Correlation coefficients between DOG1 expression and monthly climatic variables throughout the year. Minimum temperature (○), maximum temperature (◇), precipitation (□) and potential solar radiation (◊). Months on the x-axis are indicated with the first letter of the month. Blue colours indicate $P<0.05$ and no colour indicates non-significant coefficients tested by SAR models.

Discussion

Understanding the evolutionary mechanisms of plant adaptation to different environments requires the identification of the ecological factors that contribute to maintaining phenotypic variation in nature (Manzano-Piedras et al., 2014; He et al., 2014). The systematic analysis of a regional collection of 300 Arabidopsis accessions from the Iberian Peninsula carried out in this study, identified altitude and temperature as the major geographical and climatic factors associated with multiple life history traits. In particular, several results support the involvement of seed dormancy and flowering time in altitudinal and climatic adaptation in a non-independent manner. First, both traits are negatively correlated and displayed strong spatial autocorrelation, in agreement with a similar correlation described before for 112 accessions across Europe (Debieu et al., 2013). These traits showed a tendency to decrease with latitude, which resulted in the tendency of northern plants to flower later and have lower primary dormancy than southern plants (Debieu et al., 2013). Second, the natural genetic variation for seed dormancy and flowering time show strong altitudinal and temperature clines, in agreement with results of previous studies on flowering time (Méndez-Vigo et al., 2011; Manzano-Piedras et al., 2014). Third, analysis of the environmental distribution of the natural variation shows that Arabidopsis accessions displaying extreme dormant phenotypes and early flowering come from populations distributed exclusively below 1200 m altitude and from locations with a mean annual temperature higher than 9 °C. Therefore, the life cycles determined by such genetic combination seem not to be tolerated outside these environmental ranges. By contrast, the broad climatic and altitudinal distribution of accessions with low dormancy would...
and late flowering suggests that such accessions are more common and better adapted to a wider range of environments.

In addition, we found significant altitudinal and climatic clines for seed size, another important evolutionary and ecological trait for many species that is co-adapted with other life history traits (Moles et al., 2005). Small seeds can be associated with the persistence of seed in the soil for several species (Bakker et al., 1996; Bekker et al., 1998), although other studies failed to find this association (Leishman et al., 2000). It is generally assumed that seed dormancy and persistence of the seed in the soil seed bank are synonymous (Anderson, 1990; Rees, 1996; Baskin & Baskin, 1998); however Thompson et al. (2003) reported that dormancy and persistence in soil are not related. In agreement with Thompson et al. (2003), our current study on the Arabidopsis Iberian collection showed for the first time a strong negative correlation between seed size and seed dormancy as well as flowering time. Overall, these patterns suggest that populations from low altitude or warm areas flower earlier, and produce more dormant and smaller seeds than populations from higher or colder locations.

Despite the fact that the various seed dormancy-related traits appeared to be associated with the same mean annual climatic parameters, their genetic variation is affected differentially by those environmental factors, since all studied traits differ in the precise climatic patterns along the year. In particular, seed dormancy and seed size were the only life history traits significantly associated with all climatic parameters, including solar radiation during summer, of which DSDS50 showed the strongest associations. This suggests that the natural variation for seed dormancy is more sensitive to climatic components than seed size, the rate of germination or flowering time, which is in agreement with the strong plasticity of seed dormancy to numerous environmental factors (Munir et al., 2001; Alboresi et al., 2005; Kendall et al., 2011; Penfield & Springthorpe, 2012; He et al., 2014; Huang et al., 2014; Postma & Ågren, 2015). Overall, the genetic variation for seed dormancy seems affected by summer precipitation and summer solar radiation, with low precipitation and high irradiation favouring dormant genotypes. In addition, high dormancy is also associated with high temperatures along the year. A similar climatic pattern is found for the rate of germination, although with much lower strength, in agreement with the weak correlation between DSDS50 and t50. Furthermore, seed size displayed opposite climatic patterns as compared to seed dormancy, though high precipitation appeared significantly associated with larger seed size along the whole year. By contrast, flowering time showed several specific climatic associations, indicating that climate also acts on this trait. In particular, temperature had the strongest flowering association. Accessions from local populations exposed to warmer mean annual temperature flowered earlier. Precipitation in winter and summer showed opposite effect, regions with higher precipitation in winter, plants normally flowered earlier and regions with higher precipitation in summer plants flowered later, this is in agreement with previous observations (Méndez-Vigo et al., 2011; Manzano-Piedras et al., 2014).
Therefore, these results suggest that locations with low summer precipitation and high radiation, select, directly or indirectly, for life cycles with early flowering, small seeds, high seed dormancy and slow germination rate. However, other components of these climatic factors also contribute specifically to shape the geographical distribution of each life history trait.

It remains unknown how the climatic clines and associations displayed by the natural variation for seed dormancy-related traits adapt the life cycle of Arabidopsis to different environments. Taking into account the behaviour of winter and spring annual cohorts of natural populations (Probert, 2000; Donohue, 2009; Picó, 2012; Bewley et al., 2013), it might be speculated that populations from low altitudes and warm areas display a spring annual life cycle with the shortest growing period, which might be determined by strong seed dormancy and very early flowering. However, the situation is complex since ambient temperature has been shown to affect not only germination and flowering time (Fenner, 1991; Finch-Savage & Leubner-Metzger, 2006; Verhage et al., 2014), but also the induction of secondary dormancy (Penfield & Springthorpe, 2012). Moreover, a recent study showed that accessions might adopt different life strategies depending on the environmental conditions, as shown for Columbia (Col-0) which could adopt winter annual, summer annual or rapid cycling life histories (Springthorpe & Penfield, 2015). For the winter annual behaviour, earlier flowering plants produced more dormant seeds than later flowering plants, but, as a strategy of adaptation, the later flowering plants showed more rapid cycles and probably produced more seeds as descendants (Springthorpe & Penfield, 2015). In agreement with this complexity, the same authors suggested that the variation for flowering time in Arabidopsis plants with a winter annual life cycle has evolved to constraint the maternal environment for seed set to a specific temperature that coincides with a temperature-sensitive switch in the seed’s dormancy state (Springthorpe & Penfield, 2015). Interestingly, a weak but significant association was found for the low temperature response of germination with minimum temperature in summer, the season in which Arabidopsis is present only as banks of dormant seeds (Montesinos et al., 2009; Gomaa et al., 2011). Iberian accessions showing very weak germination response to low temperature appeared distributed in locations with high summer minimum temperature, suggesting that these accessions might have a higher germination-inductive temperature. Alternatively, these accessions might have lost the mechanism of germination induction mediated by low temperature. Normally dormancy can be released by several months of conservation of dry seeds in a mild/warm temperature (after-ripening) or by a low temperature treatment of imbibed seeds (cold stratification) (Bewley, 1997; Bewley et al., 2013) that induces GA biosynthesis which in turn promotes seed germination (Yamauchi et al., 2004). However, 21 accessions from our collection did not respond to cold stratification and 10 of these neither responded to after-ripening. It could be that these very dormant accessions require a different cue to germinate. Therewith, given the complex behaviour of Arabidopsis populations in nature (Picó, 2012), additional analyses under field conditions are necessary.
to relate the phenotypic variation for seed dormancy and flowering time under laboratory conditions to natural life cycles.

The correlation between DOG1 expression levels, DSDS50 and flowering time indicates that selection for these dormancy-related traits might operate via DOG1. Minimum temperature, especially in February, March and April and humanised habitat might be the driving forces of this adaptation, leading to early flowering and higher dormancy, regardless of the geographical position. The signs of DOG1 correlations (positive with DSDS50, positive with mean minimum annual temperature and negative with flowering time) are in the direction that is expected according to DOG1 as an activator of dormancy. These results, and earlier findings on structural changes in DOG1 (Kronholm et al., 2012), show how associations of a quantitative molecular phenotype and/or sequence polymorphisms allow the dissection of molecular mechanisms that might precisely fit dormancy with its environment. However, DOG1 is likely not the only gene accounting for the natural variation in seed dormancy across the Iberian population. Future studies on the genome sequences of these accessions (Cao et al., 2011; Arabidopsis 1001 genomes browser: http://1001genomes.org/) should shed light on other genes and the molecular mechanisms that underlie climatic adaptation through their effects on seed dormancy-related traits in Arabidopsis.

Conclusions

We have analysed seed dormancy levels, rate of germination, seed size and flowering time in a collection of 300 accessions from the Iberian Peninsula. By analysing spatial autocorrelation patterns of phenotypic traits and environmental variables we found that dormancy, rate of germination and flowering time are significantly and positively spatially autocorrelated. Dormancy, seed size and flowering time showed strong altitudinal clines, indicating that Arabidopsis accessions from high altitudes flower later and produce larger seeds with lower dormancy levels. High correlations of phenotypic traits and climatic parameters confirmed that dormancy, seed size and flowering time are relevant adaptive traits, and that temperature and precipitation are the main climatic factors driving the adaptation of these traits. We also showed the relevance of regulatory variation of DOG1 expression for climatic and environmental adaptation.
Supplementary Material

Supplemental files can be downloaded from:
http://www.wageningenseedlab.nl/thesis/dsvidigal

Figure S1. Schematic illustration of the primer pairs insertions in the DOG1 genomic DNA and multiple-sequence alignment of DOG1 from different accessions.

Figure S2. Altitudinal clines of rate of germination.

Table S1. Classification of the Iberian collection of Arabidopsis for flowering time: late, intermediary and early flowering.
Table S2. Seed dormancy-related traits of the 300 accessions of the Iberian collection of Arabidopsis.
Table S3. Correlations between geographical and environmental variables and seed dormancy-related traits.

Table S4. Correlations among environmental variables of the Iberian collection of Arabidopsis.

Table S5. DOG1 expression and seed dormancy-related traits of 100 accessions of the Iberian collection of Arabidopsis.
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Chapter 5

Galactinol as marker for seed longevity

Abstract

Production of high-quality seeds is the main aim of the seed industry. However, seed quality is threatened by seed deterioration. Reduced longevity or storability of seeds is recognized as a major problem contributing to increased costs of crop production. Galactinol is part of the raffinose family oligosaccharides (RFOs) pathway that is known to be involved in stress defense mechanisms, as osmoprotectants, against abiotic stresses. The RFOs have been proposed to play an important role in conferring desiccation tolerance and longevity to seeds. Studying the correlation between primary metabolites and germination phenotypes of Arabidopsis seeds, we found a positive correlation between galactinol and seed longevity. This relation seems conserved across plant species since we found the same relationship in cabbage and tomato. In order to unravel the role of the galactinol pathway in seed longevity we applied a reverse genetics approach using T-DNA knock-out lines in genes encoding enzymes of the RFO pathway (GALACTINOL SYNTHASE 1 (GOLS1), GALACTINOL SYNTHASE 2 (GOLS2), RAFFINOSE SYNTHASE (RS5), STACHYOSE SYNTHASE (STS) and ALPHA-GALACTOSIDASE (AGAL)) and overexpressors of the cucumber GALACTINOL SYNTHASE 2 gene in Arabidopsis. Of these mutants seeds of the gols2 and gols1gols2 double mutant have the lowest seed galactinol content, that coincides with a lower seed longevity. These results show that galactinol levels in the dry seed can be used as a biomarker for seed longevity.

Introduction

Orthodox seeds can be stored dry after seeds have been dispersed by the mother plant. This ability is induced during seed maturation at which physical, physiological and biochemical changes occur (Bewley et al., 2013). The final stage of the maturation process is marked by dehydration, in such a manner that during the reserve deposition phase, there is an accumulation of potentially protective molecules, especially soluble sugars, such as sucrose, raffinose and stachyose (Castillo et al., 1990; Blackman et al., 1992; Fait et al., 2006) and LEA (late embryogenesis abundant) proteins (Kermode, 1997). The LEA proteins act in synergism with the soluble sugars during cytoplasm crystallization and prevent the damage caused by water removal from the seed tissues, protecting the membrane surface (Kermode, 1997; Obendorf, 1997; Hoekstra et al., 2001).

Seed longevity represents the length of time that the seeds remain viable after reaching physiological maturity (Delouche & Baskin, 1973). Especially during dry storage, seeds lose quality due to deterioration processes, which are inevitable and irreversible. The first indicator of deterioration is a reduction of the rate of germination (which is a vigor indicator) and, consequently, at a later period of storage, the loss of germinability (Bernal-Lugo & Leopold, 1992; Bewley et al., 2013). The rate of the deterioration process is influenced by several factors, both during seed maturation when the maternal environment can influence the extension of longevity, as well as during drying and storage (Ellis & Roberts, 1980; Nagel & Börner, 2010). High light during seed maturation may notably increase seed longevity while low temperature decreases it (He et al., 2014). Moreover, high temperature and high relative humidity during the storage process, decrease seed viability and vigor significantly (Ellis et al., 1991; Vertucci et al., 1994). Seed longevity is a quantitative trait and the behavior of longevity is species- or variety specific (Nagel & Börner, 2010; Nagel et al., 2014). In order to study seed longevity in a short period of time, artificial aging tests were established, in which seeds are exposed to high temperature and high relative humidity for a short period after which germinability of the seeds is analysed (Lehle & Tanner, 1973; Hampton & Tekrony, 1995). For Arabidopsis this method has been shown to quite well mimic natural dry aging (Bentsink et al., 2000; Tesnier et al., 2002).

 Sugars in seeds have been thought to act as signals that regulate and influence seed development (Borisjuk et al., 2004). Among these sugars are the raffinose family oligosaccharides (RFO, i.e. raffinose, stachyose and verbascose) for which a role in seed longevity has been proposed. Several studies have shown that sugars help to maintain the structural integrity of membranes and proteins under dry conditions due the formation of a glassy state to limit deteriorative reactions (Bernal-Lugo & Leopold, 1995; Nishizawa et al., 2008; ElSayed et al., 2014). Alternatively, the ratio of oligosaccharides to sucrose may be used as an indicator of seed quality and storability (Bernal-Lugo & Leopold, 1992; Bernal-Lugo & Leopold, 1995; Obendorf, 1997; Bailly et al., 2001; Vandecastelee et al., 2011). However, it has also been reported that the RFO are not a good indicator of seed
vigour (Bentsink *et al.*, 2000; Bailly *et al.*, 2001) or that it is not equally applicable in all species (Horbowicz & Obendorf, 1994). So far there is no definitive proof that RFO plays a role in seed longevity.

Here we study whether galactinol can serve as a marker for seed longevity. Galactinol is produced during the first step of the RFO biosynthesis pathway where UDP-galactose is converted to galactinol by galactinol synthase (GolS), which plays a regulatory role in this pathway (Castillo *et al.*, 1990; Nishizawa *et al.*, 2008; ElSayed *et al.*, 2014). Galactinol levels are investigated in a series of near isogenic lines (NILs) and mutants that have different seed longevity. Among them the *delay of germination 1* (*dog1*) mutant which, in addition to its reduced seed dormancy, is badly storable (Bentsink *et al.*, 2006). The use of a metabolic marker as indicator of seed longevity will facilitate the seed industry in making decisions on which seed lots can be placed onto the market or can be stored for longer periods of time.

## Materials and Methods

### Plant materials

**Arabidopsis:** The twelve *Arabidopsis thaliana* genotypes (Landsberg *erecta* (Ler), Columbia (Col-0, Col), NILDOG1-Cvi, NILDOG2-Cvi, NILDOG3-Cvi, NILDOG6-Kas-2, NILDOG22-An-1, *dog1-1, dog1-3, cyp707a1-1, cyp707a2-1* and *Atnced6-Atnced9*) used by He *et al.* (2014) (Chapter 2) were also used in this study. T-DNA knock-out lines of genes encoding enzymes of the RFO biosynthetic pathway *galactinol synthase 1* (*gols1*) (AT2G47180 – SALK_046018), *galactinol synthase 2* (*gols2*) (AT1G56600 – SALK_101144), *raffinose synthase* (*rs*) (AT5G40390 – SALK_085989), *stachyose synthase* (*sts*) (AT4G01970 – SALK_045237) and *alpha-galactosidase* (*agal*) (AT5G08380 – SALK_079578) were obtained from the Nottingham Arabidopsis Stock Centre (NASC). The Arabidopsis overexpression lines of *GALACTINOL SYNTHASE 2* of cucumber (CsG26, CsG32 and CsG58 in Col-0) are describe by Zuther *et al.* (2004). The plants were grown under greenhouse conditions using Rockwool blocks (4 x 4 cm) watered with Hyponex solution, in randomized complete block design with four replicates per genotype. The T-DNA knock-out lines were screened for homozygous insertions. Homozygous mutants of *gols1* and *gols2* were been crossed in order to obtain the double mutant.

**Cabbage:** Six batches of proprietary cabbage seeds of three different varieties were obtained from Bejo Seeds BV, The Netherlands. These seeds had been produced in greenhouse conditions. The seeds were dried and size graded and subsequently stored at 20°C in paper bags. Samples were taken at different intervals during storage, and then stored at -25°C in aluminium bags until use.
Tomato: 50 recombinant inbred lines (RIL) of tomato from a cross between Solanum lycopersicum cv. Moneymaker and S. pimpinellifolium were used. The tomato seeds are from the same lots as described by Kazmi et al. (2012). In short, plants were grown under greenhouse conditions at 25°C/15°C (day/night) and long day conditions (16h light and 8h dark). After harvest the seeds were processed and treated with trisodium phosphate (Na$_3$PO$_4$.12H$_2$O) for disinfection. Seeds were dried for 3 days at 20°C and stored at 13°C and 30% relative humidity in paper bags.

Seed phenotyping

Assessment of seed performance included dormancy level (DSDS50: days of seed dry storage required to reach 50% germination), germination under temperature stress: high and low temperature (30°C and 10°C, respectively), osmotic stress (mannitol), salt stress (NaCl), abscisic acid (ABA) and artificial aging, and was executed as described in Chapter 2 for the twelve Arabidopsis genotypes.

To measure seed longevity, an artificial aging test was implemented by incubating seeds in open tubes over a saturated ZnSO$_4$ solution (40°C, 85% relative humidity) for 8 days for Arabidopsis and 15 days for tomato (ISTA, 2009). Thereafter a germination assay was performed as described in Chapter 2. For cabbage, natural aging (storage at 20°C in paper bags) was monitored, as well as the maximum germination percentage (Gmax) at several intervals during the period of dry storage.

Germination assays were executed before and after aging and Gmax and the rate of germination (t50 - time (hours) required to reach 50% germination of the total number of germinated seeds) values were extracted from the germination assay using the Germinator package (Joosen et al., 2010).

Analysis of seed metabolites using gas chromatography with time-of-flight mass spectrometry (GC-TOF-MS)

Approximately 10 mg of dry Arabidopsis seeds pre-cooled in liquid nitrogen, were homogenized in 2 ml tubes with 2 iron balls (2.5 mm) using the dismembrator (Mo Bio Laboratory, MM 400). 375 µl methanol/chloroform (4:3) was added together with 75 µl of a mixture of water and ribitol (1 mg/ml) and mixed thoroughly. After 10 minutes of sonication (Ultrasonic Branson 3510), 100 µl MQ water was added to the mixture followed by vortexing and centrifugation (5 min., 15000 rpm). The methanol phase was collected in a new 2 ml eppendorf tube. 250 µl methanol/chloroform (1:1) was added to the remaining organic phase and kept on ice for 10 min. 100 µl MQ water was added followed by vortexing and centrifugation (5 min., 15000 rpm). Again, the methanol phase was collected and mixed with the other collected phase. 50µl of the extract was dried overnight in a glass vial using a speedvac (room temperature, Savant SPD121P). 12.5 µl of O-
methylhydroxylamine hydrochloride (20 mg ml⁻¹ pyridine) was added to the sample and incubated for 30 min at 40°C with agitation. Then, the samples were derivatized with 17.5 µl of N-methyl-N-trimethylsilyl trifluoroacetamide for 60 min. An alkane mixture (C10-C18 and C20-C32) was added and from the final solution, 2 µl was injected to determine the retention indices of metabolites.

A GC-TOF-MS based method was used as described by Carreno-Quintero et al. (2012) with some minor modifications. Detector voltage was set at 1650V. Raw data was processed using the chromaTOF software 2.0 (Leco instruments) and further processes using the Metalign software (Lommen, 2009), to extract and align the mass signals. A signal to noise ratio of 2 was used. The output was further processed by the Metalign Output Transformer (METOT; Plant Research Internation, Wageningen). Centrotypes were created using the MSclust program (Tikunov et al., 2012). The mass spectra of these centrotypes were used for the identification by matching to an in-house constructed library and the NIST05 (National Institute of Standards and Technology; http://www.nist.gov/srd/mslist.htm) libraries. This identification is based on spectra similarity and comparison of retention indices calculated by using a 3rd order polynomial function (Strehmel et al., 2008).

**Analysis of sugars using high-performance liquid chromatography (HPLC)**

Sugar contents were determined as described by Bentsink et al. (2000), with minor modifications. 2 mg (for Arabidopsis) or 10 mg (for cabbage) of dried seeds were homogenized in 1 ml of methanol (80% v/v) with the addition of 20 µg of melezitose (Arabidopsis) or 100 µg of melezitose (cabbage) as internal standard. Samples were incubated in a water bath (76°C) for 15 min and the extract was dried using a Speedvac (room temperature, Savant SPD121P). The residue was resuspended in 0.5 ml MQ water (Arabidopsis) or 2.5 ml MQ water (cabbage) and thoroughly vortexed and centrifuged for 5 min at 17000 g in an Eppendorf centrifuge. The supernatant was injected into a Dionex HPLC system (ICS 5000 + DC) to analyse the sugar content, using a CarboPac PA 1, 4-× 250-mm column preceded by a guard column (CarboPac PA 1, 4 × 50 mm). Mono-, di-, and trisaccharides were separated by elution in an increasing concentration of NaOH (20–350 mM) with a flow rate of 1 ml per minute. Peaks were identified by co-elution of standards. Sugar quantity was corrected by mean of the internal standard (melezitose) and transformed to micrograms of sugar per milligram of dry material.
Correlation analysis

Correlation analysis was performed between the 38 identified primary metabolites and seed performance traits. The heat maps were constructed using the Spearman correlation coefficient matrices R-packages “MASS”, “Hmisc” and “VGAM” and the R graphic packages “gplots” and “graphics” were used for visualizing the data.

Quantitative Trait Locus (QTL) analysis

QTL analysis was performed as described by Kazmi et al. (2012). In brief, to identify the QTL positions on the genome, the mapping software MapQTL® 5.0 (Van Ooijen & Maliepaard, 2003) was used in the RIL population which contains 865 SNP markers. A multiple QTL mapping model (MQM) was used to identify potential QTLs (Jansen et al., 1995) as implemented in MapQTL® 5.0. In the final LOD profile, QTLs were affirmed according to the threshold LOD scores of 3 with 95% confidence interval (van Ooijen, 1999).

Results

Correlation analyses of seed performance and primary metabolites

To investigate whether seed performance is reflected by primary metabolite contents in dry seeds, we have investigated these metabolites for the twelve Arabidopsis genotypes for which seed performance was analysed in Chapter 2. The primary metabolites of after-ripened seeds grown under controlled conditions were analysed using untargeted GC-TOF-MS analysis. Signals of 102 primary metabolites were analysed by matching mass spectra and retention times to an in-house constructed library and the NIST05 (National Institute of Standards and Technology, Gaithersburg, MD, USA; http://www.nist.gov/srd/mslist.htm) libraries. Thirty eight metabolites could be identified, which included mainly amino acids, sugars and organic acids (Table S1).

Principal component analysis (PCA) for the primary metabolites (Fig. S1) revealed an evident separation between the two genetic backgrounds (Ler and Col). Due to this variation in genetic background, subsequent analysis was performed for each genetic background separately. Correlation analysis between primary metabolites and seed performance was used to investigate the metabolic changes in relation to the variation in seed performance traits (Fig. 1 and Table S2). The Ler and Col backgrounds showed both similar as well as distinct patterns. For example, the Col background had a strong negative correlation between dormancy level (DSDS50) and germination at high temperature ($r = -0.96$; Table S2A) whereas the Ler background did not show any correlation between these
Galactinol and seed longevity

traits ($r = 0.11$; Table S2B). Opposite directions of correlation were also found between the backgrounds. For the Ler background, correlation between dormancy and germination in ABA was positive ($r = 0.78$; Table S2B) whereas for the Col background, the correlation was negative ($r = -0.079$; Table S2A).

**Figure 1.** Correlation analysis between primary metabolites and seed performance traits. Heatmap of the Spearman correlations between the 38 identified metabolites and seed performance traits of twelve genotypes with Landsberg erecta (Ler) and Columbia (Col) backgrounds. Each square represents the Spearman correlation coefficient between seed performance (row) and metabolites (column). The colour key is indicated in the right upper corner of the figure, red squares indicate positive correlations, blue squares negative correlation and white squares no correlation. DSDS50: days of seeds dry storage required to reach 50% of germination; Gmax$_{10^\circ C}$: maximum germination at low temperature ($10^\circ C$); t50$_{10^\circ C}$: time required for 50% of viable seeds to germinate at $10^\circ C$; Gmax$_{30^\circ C}$: maximum germination at high temperature ($30^\circ C$); Gmax$_{aging}$: maximum germination after artificial aging; Gmax$_{mannitol}$: maximum germination in mannitol; Gmax$_{salt}$: maximum germination in salt (NaCl); Gmax$_{ABA}$: maximum germination in ABA.

A very strong correlation between germination after artificial aging, which is an estimation for seed longevity, and galactinol was found for both genetic backgrounds ($r = 0.91$ for Ler background and $r = 0.77$ for Col background; Table S2). In the biosynthetic pathway of the RFO, which includes galactinol, raffinose, stachyose and verbascose, myo-inositol is the compound essential for the formation of galactinol (Lehle & Tanner, 1973). Raffinose, stachyose and verbascose could not be identified in our samples but myo-inositol was. Interestingly, myo-inositol abundance correlated strongly with germination after artificial aging and galactinol, however, only for the Col background ($r = 0.81$ and 0.92, respectively; Table S2A), whereas for the Ler background this correlation was not significant ($r = 0.11$). In the remainder of this chapter we will explore the relation between seed longevity and galactinol. Among the genotypes examined, there was a dog1 mutant for
both genetic backgrounds. This mutant is badly storable (Bentsink et al., 2006) but there is a difference between the two genetic backgrounds; dog1-1 (Ler background) is less storable than dog1-3 (Col background) (Fig. 2A). This difference is reflected in the galactinol content. Col has more galactinol than dog1-3 and Ler has more than dog1-1 (Fig. 2B). Col itself contains much more galactinol than Ler, which is more sensitive to aging, illustrating very well the link between seed longevity and galactinol content (Fig. 2).

Figure 2. Correlation between seed longevity and galactinol content in Arabidopsis. A) Germination after artificial aging; B) Relative content of galactinol. Col background: Col (wild type) and dog1-3; Ler background: Ler (wild type) and dog1-1. Averages of three replicates are presented. Error bars show standard errors. Asterisks indicate significant differences between mutant and its respective wild type (**, P < 0.001; *, P < 0.05).

Confirmation in cabbage seeds

To check whether the positive correlation between galactinol and longevity, that was found for Arabidopsis, is consistent in other species as well, six batches of cabbage seeds from three different varieties: variety 1 (1.1 and 1.2) variety 2 (2.1 and 2.2) and variety 3 (3.1 and 3.2), were analysed for their longevity and galactinol content. These six batches of cabbage had been stored for several years and germination tests were performed at several intervals with the aim to determine the longevity of the seeds (Fig. 3A). The two batches of variety 3 showed the shortest longevity, after 6 years of storage both batches germinated less than 17%. For variety 1, one batch still germinated with a high percentage after 8 years of storage (batch 1.1), whereas in the other batch (1.2) germination decreased to 66% after around 6 years of storage. Both batches of variety 2 showed high germination after 6 years of storage (Fig. 3A). Galactinol contents were measured in freshly harvested
seeds (Fig. 3B) which still germinated for more than 92%, however, the variation for the rate of germination (t50) reflected the longevity of these seeds (Fig. 3C). A negative correlation between t50 and relative galactinol content was found (r = -0.61). The batches of variety 3 had the lowest longevity (Fig. 3A), the highest t50 in freshly harvested seeds (Fig. 3C) and the lowest seed galactinol content (Fig. 3B). Thus, also for cabbage a positive correlation between seed longevity and galactinol content could be identified.

Figure 3. Seed longevity and galactinol content in cabbage. A and D: Six batches of cabbage seeds stored for several years. B and C: Six batches of fresh harvested cabbage seeds. A) Germination (%) over the years of storage; B) Relative content of galactinol of fresh harvested seeds; C) t50 (time (hours) required to reach 50% germination of the total number of germinated seeds) of fresh harvested seeds; D) Relative content of galactinol in three time points during the storage period. Variety 1: 1.1 and 1.2; Variety 2: 2.1 and 2.2; Variety 3: 3.1 and 3.2. Averages of three replicates are presented. Error bars show standard errors. Means followed by the same letter did not differ by Tukey's test (P<0.05).

Galactinol content, at three time points during dry storage (Fig 3D), was measured to determine whether the galactinol concentration was affected by dry storage and therefore correlated with seed longevity. However, galactinol content did not change over the years of storage and this result was consistent for all batches. Thus, the initial galactinol levels correlate with seed longevity and the batches with the lowest amounts of galactinol (variety 3, Fig 3A) are the ones with the lowest seed longevity (Fig. 3D).
Confirmation in tomato seeds

The link between longevity and galactinol content was also checked in seeds of tomato *Solanum lycopersicum* cv. Moneymaker and *Solanum pimpinellifolium*. A similar positive correlation between germination after artificial aging and content of galactinol was found (Fig. 4A and B, respectively). Moneymaker is more sensitive to aging (Fig. 4A) and the galactinol content (Fig. 4B) is lower than that of *S. pimpinellifolium*.

Due to the availability of the RIL population between *S. lycopersicum* cv. Moneymaker and *S. pimpinellifolium*, the genetic basis of the correlation between seed longevity and galactinol content could be inspected. QTL analyses for seed longevity measured as germination capacity after artificial aging led to the identification of two main QTLs (Fig. 4C), one on chromosome 2 and one on chromosome 6. QTL analyses for galactinol content in the same population led to the identification of one main QTL on chromosome 2 (Fig. 4C), which coincides with the QTL for germination after artificial aging. Under this QTL there is a *GALACTINOL SYNTHASE* gene (Solyc02g084980.2.1) located.

Confirmation by T-DNA knock-out and overexpressor lines of *GALACTINOL SYNTHASE*

The correlation between seed longevity and galactinol content seems to be consistent across different species; i.e. Arabidopsis, cabbage and tomato. To obtain more insight in this correlation, T-DNA knock-out lines for genes of the RFO pathway (*gols1*, *gols2*, *rs*, *sts*, *agal* and *gols1gols2* double mutant) and overexpressors of the cucumber *GALACTINOL SYNTHASE* 2 gene were investigated for their sugar contents and longevity (Table S3). Seed galactinol levels of the *gols2* mutants and the *gols1gols2* double mutant were significantly lower and those of the *rs* and *sts* mutants significantly higher than those of wild type Col (Fig. 5A, Table S3). The galactinol levels of the other mutants did not differ from Col (Fig. 5A, Table S3). It remains unclear why galactinol levels in *gols1* seeds are unaffected, since the mutant was a true knock-out as was concluded from qRT-PCR analyses (see Supplemental Material 1; Fig. S2). Although the *GALACTINOL SYNTHASE* expression in the overexpressors was increased (Fig. S2), this did not result in higher galactinol levels in the seeds. Probably other steps in the pathway limit higher galactinol production. The other sugars (e.g. raffinose and stachyose) also did not show significant difference between the mutants (*rs* and *sts*) and the wild type.
Figure 4. Seed longevity and galactinol content in tomato. A) Seed longevity of the parents *S. lycopersicum* cv. Moneymaker (Money) and *S. pimpinellifolium* (Pimp); B) Relative seed galactinol content of Money and Pimp; C) QTL mapping for germination after artificial aging and seed galactinol content using Recombinant Inbred Lines derived from a cross between *S. lycopersicum* cv. Moneymaker and *S. pimpinellifolium*. Logarithm of Odds (LOD) scores indicate the strength of the QTL. QTL scores above LOD 3 can be considered significant.

To assess whether the altered galactinol levels affected seed longevity, germination was investigated on just after-ripened seeds after 8 days in high temperature (40°C) and a high relative humidity (85%). The mutants *gols2* and *gols1gols2* were the only mutants that showed significantly reduced seed longevity, when compared to Col (Fig. 5B). Germination after artificial aging of the *rs* and *sts* mutant did not differ significantly from wild type Col (Fig. 5B).

**Discussion**

Production of high-quality seed is the main aim of the seed industry; however the deterioration of the seeds during storage is a substantial problem which contributes to increased costs of crop production. The seed industry is seeking for information and techniques to eliminate or at least minimize seed storage losses. Most of the time these
losses are related to harvest and drying processes together with storage conditions (Ellis & Roberts, 1980; Nagel & Börner, 2010). A fast identification of which seed lots could be stored for longer time or which ones need to go immediately to the market would help the industry to make better decisions and avoid losses by storage.

![Figure 5](image_url)

**Figure 5.** T-DNA knock-out mutants of the RFO pathway and overexpressors of *GALACTINOL SYNTHASE 2*. A) Galactinol content in seeds; B) Seed longevity. Wild type (Col); T-DNA knock-out mutants: gols1 (*galactinol synthase 1*), gols2 (*galactinol synthase 2*), rs (*raffinose synthase*), sts (*stachyose synthase*), agal (*alpha-galactosidase*) and dm (*gols1gols2 double mutant*); overexpressors of the cucumber *GALACTINOL SYNTHASE 2* gene: o.e.26 (CsG26), o.e.32 (CsG32) and o.e.58 (CsG58). Averages of four replicates are presented. Error bars show standard errors. Asterisks indicate significant differences between mutant and the wild type (**, *P* <0.001; *, *P* <0.05).

Several biochemical tests have been used as indicator of seed quality, such as tetrazolium (Hampton & Tekrony, 1995), electrical conductivity (Milosevic et al., 2010), enzyme activity (Ramiro et al., 1995), ethylene (Siriwitayawan et al., 2003), volatile compounds (Zhang & Roos, 1997), ratio of sucrose:RFO (Bernal-Lugo & Leopold, 1992; Bernal-Lugo & Leopold, 1995; Steadman et al., 1996; Vandecasteele et al., 2011).
Metabolites have a great potential to serve as a marker since it is relative easy to develop diagnostic tests for their detection. Several studies have investigated the relation between phenotypes and metabolic profiles (Fiehn, 2002; Fait et al., 2006; Toubiana et al., 2012; Carreno-Quintero et al., 2012; Joosen et al., 2013), however up to now there is a lack of data on the relation of primary metabolites with seed performance, especially seed longevity. In the present work we investigated metabolic variation in relation to differences in seed longevity of Arabidopsis, cabbage and tomato.

In Arabidopsis a clear distinction in metabolite profiles between the genetic backgrounds Ler and Col was found. Since it is known that the erecta gene is affecting many traits (van Zanten et al., 2009) we have investigated whether it is also the erecta gene that is causing the difference between the two. For that several genotypes which included mutants and complementation lines containing either the wild type or mutated form of erecta (see Supplemental Material 2) were grown and metabolites were analysed. The PCA for the metabolites did not show differences for the erecta gene, but did show an effect of the background of the genotypes (Ler and Col) (Fig. S3). The difference between these backgrounds might be related to the adaptive properties of the genotypes, since Ler originates from northern Europe and Col from Columbia, USA (Koornneef & Meinke, 2010). In addition, also similarities were identified in the Ler and Col backgrounds, among which a strong and positive correlation between germination after artificial aging (indicator of seed longevity) and galactinol content. Changes in soluble sugar contents may contribute to reduced vigor and germinability of seeds (Bernal-Lugo & Leopold, 1992). Sucrose is the most highly abundant sugar in maize seeds, but its presence does not correlate with better storage; however raffinose as a mass fraction of total sugars showed a high and positive correlation with longevity in that species (Bernal-Lugo & Leopold, 1995). Raffinose is known to support sucrose as a membrane integrity protector by limiting lipid crystallization and deteriorative reactions (Williams & Leopold, 1989; Leprince et al., 1993). During aging the glassy state of the membranes can be lost and this enhances the degree of deterioration of the seeds (Sun & Leopold, 1993). In our assays, sucrose content also did not correlate with seed longevity. However, myo-inositol, which plays a role in the biosynthesis of oligosaccharides (Lehle & Tanner, 1973) was found to be correlated with seed longevity but only in genotypes with Col background.

The accumulation of sugars during maturation varies across species. For example, in Arabidopsis raffinose and stachyose accumulate very late in seed maturation, while verbascose is not present (Baud et al., 2002). Horbowicz & Obendorf (1994) showed that maize accumulates raffinose but not stachyose; lettuce accumulates raffinose, but not stachyose and verbascose and castor bean accumulates raffinose, stachyose but not verbascose, whereas galactinol and myo-inositol are present in seeds of several different species. The seeds of the badly storable Arabidopsis abscisic acid insensitive 3-5 mutant do not accumulate raffinose and stachyose in substantial amounts but, they did accumulate high levels of sucrose during maturation. A correlation between the ratio of mono-
oligosaccharide and longevity cannot be excluded according to Ooms et al. (1993). In our data, the correlation between seed longevity and galactinol is persistent in three species, Arabidopsis, cabbage and tomato.

During storage, seed vigor and viability are affected (Bernal-Lugo & Leopold, 1992; Nagel & Börner, 2010; Bewley et al., 2013), as was observed for the cabbage seeds in our analyses. Germination of some batches decreased after several years of storage. The galactinol content in the seeds did not change over the storage time, which can be explained by the lack of metabolic activity in dry seeds. RFOs and galactinol accumulate during the first half of seed maturation (Bailly et al., 2001; Peterbauer et al., 2001; Lahuta et al., 2005; Obendorf et al., 2009) but whether there are also changes during storage remains elusive.

Bernal-Lugo & Leopold (1992) observed that the levels of several sugars gradually declined during accelerated aging of maize seeds. Of these raffinose was the most closely associated with the decline in vigor. However, contradictory results were found by Pukacka et al. (2009), who measured the levels of sugars (sucrose, raffinose and stachyose) of beech seeds during 12 years of storage. They observed that the levels of sucrose and stachyose changed during storage but only after 7 years of storage (sucrose increased and stachyose decreased), and raffinose did not change.

By performing QTL mapping for germination after artificial aging and galactinol content in a tomato RIL population derived from a cross between *S. lycopersicum* cv. Moneymaker and *S. pimpinellifolium*, we identified two major QTLs for germination after artificial aging (chromosome 2 and 6) and one QTL for galactinol content (chromosome 2). The QTL for galactinol content co-located with the QTL for germination after artificial aging on chromosome 2, under which a *GALACTINOL SYNTHASE* gene (Solyc02g084980.2.1) is positioned. This might indicate that allelic differences in GOLS affect the galactinol content and by that seed longevity.

So far we could confirm the correlation between seed longevity and galactinol in three species: Arabidopsis, cabbage and tomato. To achieve a better comprehension of this correlation, knock-out mutants of the RFO pathway, as well as overexpressors of the cucumber *GALACTINOL SYNTHASE* 2 gene were phenotyped for seed longevity and sugar content. *GALACTINOL SYNTHASE* is an important enzyme in the regulation of the pathway toward synthesis of RFO. The activity of this enzyme is increased during seed maturation (Castillo et al., 1990), the same period when seeds acquire high vigor (Bewley et al., 2013). In Arabidopsis there are seven members of the AtGOLS gene family, of which only AtGOLS1 and AtGOLS2 were detected in mature seeds (Taji et al., 2002). The gols2 mutant and the gols1gols2 double mutant showed reduced amounts of galactinol that coincided with a decreased seed longevity (Fig. 5, Table S3), while the galactinol level in gols1 was unchanged. We expected AtGols1 and AtGols2 to function in a similar way, and would therefore expect similar phenotypes for both knock-out mutants. Both genes are highly homologous, although differences might be caused by the putative serine phosphorylation site that is present in the GOLS1 but not in GOLS2. Further they differ in
expression level, as GOLS2 was higher expressed than GOLS1 (Taji et al., 2002). It might be due to the higher GOLS2 expression that a mutation in this gene did result in altered galactinol levels while the gols1 mutant did not have an altered galactinol level. The sts mutant was the genotype with highest amount of galactinol, which is in agreement with Horbowicz & Obendorf (1994), who show that mutants with low stachyose amounts in soybean showed high amounts of galactinol, indicating that after the galactinol synthase step, the pathway is blocked. The higher galactinol levels in the sts mutant did, however, not lead to better storable seeds, probably other factors like the reduced stachyose were preventing improved seed longevity.

Conclusions

A positive correlation between galactinol and seed longevity was conserved across plant species since it was confirmed in Arabidopsis, cabbage and tomato seeds. Seed galactinol content did not change during long-term storage of cabbage, which can be explained by the fact that galactinol accumulates during seed maturation whereas during seed storage metabolic activity is low. Reduction of the galactinol content, in mutants, resulted in a reduced seed longevity (gols2 and gols1gols2). Although increased galactinol levels (rs and sts) did not improve seed longevity, galactinol may be a suitable bio-marker of seed longevity, which may be of benefit to the seed industry in making decisions on which seed lots can be put in the market or can be stored for longer periods of time.

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Supplementary Material

Supplemental files can be downloaded from:
http://www.wageningenseedlab.nl/thesis/dsvidigal

Table S1. All the 102 detected primary metabolites abundance of 12 genotypes.

Table S2. Correlation analysis between seed performance traits and the 38 metabolites identified. A) Col background; B) Ler background.
Table S3. Sugar analysis using HPLC of knock-out mutants of the RFO pathway and overexpressors of GALACTINOL SYNTHASE 2.

Table S4. All the 145 detected primary metabolites abundance of 5 genotypes included mutants and complementation lines containing either the wild type or mutated form of *erecta*.

Figure S1. Principal component analysis (PCA) of all the 102 detected metabolites of the 12 genotypes of Arabidopsis.

Figure S2. Gene expression of *GOLS1* and *GOLS2*.

Figure S3. Principal component analysis (PCA) of all the 145 detected metabolites of the 5 genotypes included mutants and complementation lines containing either the wild type or mutated form of *erecta*.

Supplemental Material 1: qPCR of *GOLS1* and *GOLS2*.

Supplemental Material 2: *Erecta* experiment.
References


Galactinol and seed longevity


Chapter 6

General Discussion
Plant adaptation is a feature that allows plants to live in a particular place or habitat. To ensure the survival of the species, plants need to acclimate to the changes in the environment. This may have a cost for the offspring and may result in lower amounts of seeds, smaller seeds, lower seed longevity and also produce seedlings with lower vigor, as well as longer germination times, as changes in the environment may promote seed dormancy. In this thesis, I have shown that seed performance is extremely sensitive to the maternal environment during seed development, that plants can adapt to environmental perturbations and that that can be highly relevant for the offspring, providing an adaptive advantage.

Effect of the environment on seed performance

Seed performance is determined by a combination of physical properties, viability, vigor, uniformity, dormancy and longevity (Basra, 2006) and it is a complex adaptive trait that is influenced by interactions among multiple genetic- and environmental factors (Koornneef et al., 2002). Seed quality is acquired during seed development and maturation and, therefore, unfavourable environmental conditions during these stages, including non-optimal temperature and light, drought stress and lack of nutrients, may reduce seed performance (Basra, 1995; Basra, 2006; Bewley et al., 2013).

In this thesis I used three different strategies to investigate the influence of adverse environmental conditions on seed performance in Arabidopsis thaliana (Arabidopsis). First, seeds of a set of different genotypes: two wild types, Landsberg erecta (Ler) and Columbia (Col-0), five Near Isogenic Lines (NILDOG1, NILDOG2, NILDOG3, NILDOG6, NILDOG22) that are known to be affected in seed dormancy (Bentsink et al., 2010), as well as two mutants with a lesion in the DELAY OF GERMINATION (DOG1) gene (dog1-1 in Ler background and dog1-3 in Col-0 background), and mutants that affect ABA biosynthesis (Atnced6Atnced9 double mutant (Lefebvre et al., 2006)) and catabolism (cyp707a1-1 and cyp707a2-1 (Kushiro et al., 2004)) were produced under different maternal growth conditions (with as variables light intensity, photoperiod, temperature, nitrate, phosphate and the interaction between phosphate and temperature). Plant and seed performance were studied (Chapter 2). Second, in Chapter 3 I have subjected all the non-transgenic genotypes that I used in the above experiment, to a field experiment. Seeds were germinated and grown into plants in different seasons of the year (summer, autumn, winter and spring) to analyze the performance of seeds that had developed in a natural environment. Third, I studied seed dormancy, rate of germination, seed size and flowering time, using a collection of 300 Arabidopsis wild accessions that were collected from different environments within the Iberian Peninsula. Spatial autocorrelation patterns of environmental variables and phenotypic traits (among which seed dormancy and seed size), as well as the relationship between environmental and phenotypic data, were used to obtain
a better insight in the effect of the environment on the evolution of the regulation of seed dormancy-related traits (Chapter 4).

**Adaptation and acclimation in response to environmental factors**

The naturally occurring genetic variation for seed performance suggests that this trait is involved in the adaptation to different climatic factors. Acclimation or phenotypic plasticity can be defined as a change in the phenotype expressed by a single genotype in different environments (Gratani, 2014; El-Soda et al., 2014). When phenotypic plasticity differs between genotypes, this is called genotype by environment interaction (GxE) (Assmann, 2013; El-Soda et al., 2014).

GxE interactions have been identified frequently for many phenotypic traits (Weigel, 2012; Assmann, 2013; El-Soda et al., 2014; El-Soda et al., 2015), including seed related traits, as described in Chapter 2 and Chapter 3. In Chapter 2, highly significant GxE interactions were found. For example, for seed dormancy it was observed that the genotypes with higher dormancy levels (such as NIL-DOG1, NIL-DOG3, NIL-DOG6 and cyp707a1-1) displayed higher plasticity in low nitrate or at low temperature than less dormant genotypes. That is, the former genotypes displayed a stronger increase in dormancy under low nitrate or low temperature. In Chapter 3 most genotypes showed similar responses to the different growth seasons; however NIL-DOG2, which is known to flower slightly earlier than wild type Ler due to the presence of the CRYPTOCHROME 2 (CRY2) Cape Verde Islands (Cvi) allele (El-Assal et al., 2001; Alonso-Blanco et al., 2003), showed a strong interaction with the environment. NIL-DOG2 sown in spring produced seeds with the lowest dormancy level, followed by the seeds of NIL-DOG2 sown in winter, autumn and summer, respectively.

Genetic variation for maternal photoperiod and seasonal dormancy in a population of Arabidopsis recombinant inbred lines (RILs) was reported by Munir et al. (2001). They demonstrated that both the maternal photoperiod and stratification of the progeny influenced seed germination and that the expression of maternal photoperiod effects is dependent on the progeny stratification. High temperature during seed development decreased seed dormancy and seed yield in two accessions of Arabidopsis (Burren (Bur) and Cvi) in comparison with plants grown at low temperature. However Bur plants were more sensitive to high temperature than Cvi plants. Bur produced less seeds but with lower dormancy as compared to Cvi when both accessions were grown at high temperature (Huang et al., 2014).

In Chapter 4, using a collection of 300 Arabidopsis accessions from the Iberian Peninsula, I studied the influence of geographic and environmental variation on adaptation. Seed dormancy, rate of germination and flowering time were found to be significantly spatially autocorrelated with latitude and longitude. Furthermore, at high altitudes, plants likely flower later and produce larger seeds with lower dormancy level.
Temperature as the key factor for seed performance

Global temperatures have risen over the past decades and, as a result, prolonged dry periods are becoming more frequent. These environmental changes may strongly influence the physiology, behavior, abundance and distribution of many species of animals and plants (Franks & Hoffmann, 2012). Temperature was found to be an important environmental factor affecting not only plant growth but also seed quality (Grass & Burris, 1995). Higher temperatures during seed development reduce seed yield and increase protein content of *Lupinus angustifolius* (Jansen, 2008). Low temperatures during seed development promote primary dormancy, whereas warm temperatures reduce dormancy, as described for many species, including *Beta vulgaris*, *Lactuca sativa*, *Amaranthus retroflexus*, *Avena fatua* (Fenner, 1991) and Arabidopsis (Schmuths et al., 2006; Kendall et al., 2011; Chiang et al., 2011; Penfield & Springthorpe, 2012; Kendall & Penfield, 2012; Chiang et al., 2013; Huang et al., 2014; Springthorpe & Penfield, 2015). My findings described in Chapters 2, 3 and 4 support the notion that temperature is indeed the most important maternal environmental cue to influence seed performance. However, other factors such as water availability, which are also known to affect germination and growth rates (Bewley et al., 2013), were not studied in Chapters 2 and 3. By manipulating only the temperature during seed development I found that low temperature in the climate room (15°C) prolonged the reproductive period of the Arabidopsis plants, increased the number of siliques per plant, and resulted in larger seeds that were more dormant, more sensitive to artificial aging (longevity) as well as to osmotic and salt stress (Chapter 2). However, growing the same genotypes in the field (Chapter 3) in The Netherlands (51°59’05.7”N, 5°39’29.2”E, 25m above sea level), I observed that plants that were grown during the summer (harvest in November 2012), when temperatures begin to drop, to below an average of 13°C, produced seeds with deeper dormancy and of smaller size. This opposite effect of temperature on seed size among these experiments can likely be explained by the fact that the growth chamber had a constant temperature, whereas under field conditions temperatures normally fluctuate (ranging from minimum 5°C to maximum 22°C during seed development). In addition, the combination of different environments, such as photoperiod, water supply and nutrients, were controlled in the climate room but not in the field experiment.

Maternal temperature may affect seed size and, normally, low temperatures during seed development are associated with greater seed weight and larger seeds (Fenner, 1992). For example, lupin (*Lupinus albus* L.) plants that were grown in chambers at 13°C or 28°C during seed development produced greater mass per seed at the cooler maturation temperature (Clapham et al., 2000). Elevated temperatures during seed development of red kidney bean resulted in smaller seeds (Thomas et al., 2009). In general, elevated temperatures increase the rate of ripening and reduce the period of time available for the accumulation of photosynthetic assimilates by the seed, consequently reducing the seed size.
or mass (Fenner, 1992). However, increasing temperatures during growth of *Leymus chinensis* plants increased the proportion of heavy-weighted seeds (Gao *et al.*, 2012). This difference in results must be caused by differences in environmental factors, since, normally, the grain filling stage of *L. chinensis* occurs when temperature and rainfall are at their highest, but in that study an increase of the temperature of only 1.7°C apparently did not constrain seed development. Therefore, I hypothesize that the temperature amplitude is an important factor for the production of smaller or larger seeds, and probably the exact stage during seed filling at which the seeds experience the temperature changes.

Environmental data from the location of progenitor accessions of the Iberian Peninsula population clearly demonstrated that, again, temperature is one of the key environmental cues in relation to adaptation of seed dormancy, seed size and flowering time, together with precipitation and altitude (*Chapter 4*). Temperature correlated positively with dormancy and negatively with seed size, and precipitation during summer correlated negatively with dormancy and positively with seed size. This means that accessions which are established at warmer and drier places of the Iberian Peninsula, are expected to produce smaller seeds with deeper dormancy. In this type of environment, germination is very risky due to the aridity of the summer season, which explains the presence of more dormant seeds in accessions from these regions. Using a subset of the same Arabidopsis population, Montesinos-Navarro *et al.* (2011) and Manzano-Piedras *et al.* (2014) observed that seed weight is higher in plants from locations with low temperatures and high precipitation. According to these authors, seed weight can be linked with the variation in flowering time, since the life cycle of early flowering is shorter and, with that, the amount of resources translocated from the mother plant to the seeds is lower, resulting in lighter seeds. These observations corroborate the conclusions of *Chapter 4*, namely that high temperatures with low precipitation induce early flowering, small seeds and deep dormancy. The precise mechanism by which temperature affects seed dormancy remains unclear. However, it might depend on epigenetic signals, as was suggested for *Flowering Locus T* which controls dormancy of the progeny by the temperature experienced by vegetative tissues of the mother plant, before flowering (Chen *et al.*, 2014).

**DOG1 influences seed dormancy in response to environmental changes**

The environment appears to affect seed dormancy through one of the main dormancy regulators, **DOG1**. **DOG1** is the main quantitative trait locus (QTL) that is associated with natural variation in seed dormancy of Arabidopsis (Alonso-Blanco *et al.*, 2003; Bentsink *et al.*, 2010), and the first one that is cloned (Bentsink *et al.*, 2006). **DOG1** appears not only important for dormancy under laboratory conditions since also QTL analyses for field germination phenology identified the **DOG1** locus (Huang *et al.*, 2010). **A**
study using transcriptome comparisons of Arabidopsis grown under high- and low
temperatures indicated that low temperatures during seed maturation induced the activity of
several genes related to dormancy, including DOG1 (Kendall et al., 2011). Furthermore, a
study using different sets of Arabidopsis accessions from Eurasia and Africa also showed
that DOG1 expression was associated with temperature during seed maturation; seeds that
matured at low temperature displayed an increase in both dormancy and DOG1 expression
(Chiang et al., 2011). These authors also showed that southern accessions are normally
more dormant and have higher DOG1 expression levels during seed maturation than
northern accessions. Kronholm et al. (2012) showed that DOG1 contributes to local
adaptation when they evaluated 289 individuals collected in 41 populations from four
geographically separated regions (Spain, France, Norway and Central Asia). A recent study
suggests that DOG1 indeed affects primary dormancy induced by the temperature during
seed development, but also controls secondary dormancy, when imbibed seeds are exposed
to prolonged warm or cold stratification. The effect of DOG1 is stronger when secondary
dormancy is induced by cold stratification than induced by warm seed stratification
(Murphey et al., 2015). In Chapter 4, I showed that earlier flowering and higher dormancy
are likely mediated by DOG1 expression, which may be part of a mechanism to adapt to
minimum annual temperature and humanised habitat.

DOG1 does not only play a role in seed dormancy but also in seed longevity
since transgenic lines containing the DOG1-Cvi allele in the Ler genetic background were
both more dormant and less storable than Ler (Nguyen et al., 2012). In Chapter 2, I
showed that also the seed maturation environment can affect seed dormancy and seed
longevity in the same manner, as NILDOG1-Cvi seeds derived from low maternal
temperature and low nitrate concentrations, were more dormant and less storable.
Moreover, DOG1 was found to influence not only seed dormancy and seed longevity but
also flowering time, since seeds of NILDOG1-Cvi that were dispersed in autumn (cold
period) germinated only the next spring, which caused the acceleration in flowering time of
these spring germinated seeds (Chiang et al., 2013). Thus, flowering time and seed
dispersal are indirectly interdependent, since flowering time determines when the seeds are
dispersed and seed dormancy regulates germination of the next generation and, hence,
flowering time (Donohue et al., 2005).

Knowledge transfer to crops

Seed performance, especially seed dormancy, is highly dependent on the
environment during seed development. High quality seed production is the aim of all seed
companies, since it ensures optimal seedling performance in the field for the farmers.
Methods to enhance seed performance have been developed, of which the most widely used
is seed priming. This is a prehydration treatment of dry seeds to advance their germination
to a certain extent, followed by dehydration and storage of the seeds until sowing
A priming treatment results in a higher rate of germination, more uniform emergence and gives better yields in vegetable, floriculture and field crops (Taylor & Harman, 1990; McDonald, 2000). It is also suggested that priming is an effective method to enhance stress tolerance of plants to adverse environments (Jisha et al., 2013). However, a negative side effect of seed priming is that it reduces seed longevity (Argerich et al., 1989; Tarquis & Bradford, 1992; Liu et al., 1996; Chiu et al., 2002). Seed longevity (storability) is a major problem in agricultural production and the maternal environment during seed maturation may influence longevity, as well as methods of drying and storage (Ellis & Roberts, 1980; Nagel & Börner, 2010). I have shown that high light during seed maturation may increase seed longevity considerably whereas low temperature decreases it (Chapter 2).

During storage, seed vigor and viability decrease (Bernal-Lugo & Leopold, 1992; Nagel & Börner, 2010; Bewley et al., 2013). High temperature and high relative humidity during storage accelerate this process of deterioration (Ellis et al., 1991; Vertucci et al., 1994; Bewley et al., 2013). Changes in soluble sugar content may contribute to this reduced vigor and germinability (Bernal-Lugo & Leopold, 1992; Bernal-Lugo & Leopold, 1995; Steadman et al., 1996). Sugars are important compounds for survival and plants are able to continuously sense sugar levels and control their energy status (Lastdrager et al., 2014).

During the middle to late stages of maturation seeds accumulate various types of sugars, such as the raffinose family oligosaccharides (RFOs) and sucrose (Handley et al., 1983; Lahuta et al., 2005; Obendorf et al., 2009). Raffinose is known to support sucrose as a membrane integrity protector by limiting lipid crystallization and deteriorative reactions, thus helping to slow down the aging process (Williams & Leopold, 1989; Leprince et al., 1993). **Galactinol Synthase (GOLS)** is the first enzyme in the RFO biosynthetic pathway, converting UDP-galactose to galactinol, and plays a role in the regulation of the pathway (Castillo et al., 1990; Nishizawa et al., 2008; ElSayed et al., 2014). RFOs are known to play a role in the acquisition of desiccation tolerance during seed maturation by protecting cellular integrity by stabilizing membranes during dehydration (Horbowicz & Obendorf, 1994; Obendorf, 1997; Li et al., 2011). RFOs are also involved in stress defence mechanisms (Bartels & Sunkar, 2005; ElSayed et al., 2014). Accumulation of raffinose and galactinol is involved in tolerance to drought, to high salinity and to cold (Taji et al., 2002). The latter authors also reported that GOLS genes (AtGOLSI, 2 and 3) display different stress-responses to drought, high salinity or cold. Galactinol and raffinose were also found to be scavengers of reactive oxygen species (ROS) under stress conditions (Nishizawa et al., 2008). However, to date not much is known about the function of galactinol besides its involvement in the production of RFOs.

In addressing the question whether seed performance is reflected by primary metabolite contents in dry seeds, I found an interesting positive and strong correlation between galactinol and seed longevity in different genotypes of Arabidopsis. This
correlation was conserved over plant species since it was also confirmed in cabbage and tomato seeds (Chapter 5). To study seed longevity in a short period of time, artificial aging was performed for Arabidopsis and tomato, in which seeds were exposed to high temperature (40°C) and high relative humidity (85%) for several days, whereas for cabbage, data of natural aging (stored at 20°C for several years) was used. So far, this method of artificial aging has been shown to mimic well the natural aging of Arabidopsis (Bentsink et al., 2000; Tesnier et al., 2002). Using both methods, artificial aging (Arabidopsis and tomato) and natural aging (cabbage), I demonstrated that a correlation between galactinol content and seed longevity is likely. Seed galactinol content did not change during long-term storage of cabbage, which can be explained by the fact that galactinol accumulates during seed maturation whereas during seed storage metabolic activity is virtually absent. Therefore, galactinol is a potential bio-marker of seed longevity which may be of benefit to the seed industry in making decisions on which seed lots can be put in the market or can be stored for longer periods of time.

Concluding remarks

This thesis shows that the maternal environment is very important for acclimation (short term) and adaptation (long term) of seed performance, especially seed dormancy. Experiments in climate chambers can be used to identify the environmental factors that affect seed performance; however, I have shown that these findings not always can be confirmed in natural environments. This shows the importance of performing field experiments in order to be able to transfer results to practice. In the field the combination of environmental factors or the greater extent of fluctuations (e.g. temperature and precipitation) may lead to different results. These findings together with the identification of a biomarker for seed longevity will facilitate seed researchers and the seed industry to improve seed quality, since they have now the possibility to test if different species respond in the same way to different environments and after that be able to select the most optimal growing location to produce seeds of high quality.
References


Climate change has a profound effect on plants and could even lead to the extinction of a species. However, plants have different strategies to survive in harsh environments and one of these is survival as a seed. Studying the interaction between genotype and environment can provide a better insight in how the environment may influence seed performance. In Chapter 2, twelve genotypes of Arabidopsis thaliana of which seeds had developed in different environments (standard conditions, low and high light, short day, continuous light, low and high temperature, low and high nitrate, low and high phosphate) were phenotyped for 18 plant and seed performance traits. Temperature played a dominant role in plant and seed performance, while light intensity and photoperiod only had an impact on plant performance. The nutrients, different amounts of nitrate and phosphate, provided to the mother plant had the least influence on plant and seed performance. Highly significant genotype by environment (GxE) interactions were found in this study. For example, for seed dormancy, an interaction was identified of the deeper dormancy genotypes - such as the Near Isogenic Lines (NILs) of the Delay of Germination (DOG) genes NILDOG1, NILDOG3, NILDOG6 and a mutant that is affected in ABA catabolism (cyp707a1-1) - with low nitrate and low temperature. These genotypes interacted with low nitrate or low temperature, leading to higher dormancy levels.

To explore the effect of environmental changes on seed performance, all the non-transgenic genotypes that were used in Chapter 2 were also grown in the field (Chapter 3). Seeds were sown in summer, autumn, winter and spring, and seed performance of the progeny was analyzed. Soil temperature, air temperature and day length were monitored during this study. Comparisons between the experiment performed under controlled conditions (Chapter 2) and the field conditions (Chapter 3) were made. When the days became shorter and the temperatures started to decrease (summer experiment), plants produced smaller seeds that were more deeply dormant. Differences were found between field and controlled conditions, since seeds produced under control conditions were larger with lower dormancy as compared to all four seasons of growth in the field (Chapter 3). Comparisons of plants grown in different environments (Chapter 2) and plants grown in a natural environment (field) (Chapter 3) indicated that indeed the temperature experienced during seed development plays a very important role in seed performance, especially in relation with seed dormancy. Although the photoperiod alone did not have any significant effect on seed performance (Chapter 2), the interaction between environments, as occurring in the field (photoperiod and temperature) led to variation in seed dormancy and seed size (Chapter 3). In Chapter 3 I observed that seed dormancy is largely controlled by the environment since the differences caused by the different seasons were larger than the differences among the genotypes with one exception. NILDOG2, which is known to flower slightly earlier than wild type Landsberg erecta (Ler) due to the presence of the CRYPTOCHROME 2 (CRY2) Cape Verde Islands (Cvi) allele, was the only genotype that
showed a strong interaction with the environment. NILDOG2, sown in spring, produced seeds with the lowest dormancy level, followed by the seeds of NILDOG2 sown in winter, autumn and summer, respectively.

In Chapter 4, the phenotypic plasticity of seed dormancy-related traits (i.e. seed dormancy, rate of germination, seed size and flowering time) and the effects of geographic and environmental variation in a regional collection of 300 natural accessions of Arabidopsis from the Iberian Peninsula were studied. Dormancy, rate of germination and flowering time were found to be significantly spatially autocorrelated with latitude and longitude, indicating that populations that are located geographically closer, are genetically more similar for these traits. Strong altitudinal clines were found for seed dormancy, seed size and flowering time, which means that at low altitudes, plants likely flower earlier and produce smaller seeds with deeper dormancy. Furthermore, seed dormancy and seed size were the only traits significantly associated with all climatic parameters, including solar radiation during summer, with dormancy showing the strongest associations. Dormancy showed a positive correlation with minimum and maximum temperature over the year and solar radiation during summer, but negative correlation with precipitation in spring and summer seasons. Seed size showed similar correlation patterns over the year as dormancy, but with opposite signs for temperature, precipitation and solar radiation. Flowering time was negatively correlated with temperature and precipitation during autumn and winter and no correlation was found with solar radiation. This implies that accessions which are established at warmer and drier places of the Iberian Peninsula, are expected to flower earlier and produce smaller seeds with higher dormancy. In Chapters 2, 3 and 4 I showed the importance of the environment on seed performance and that temperature is one of the key environmental cues playing a role in seed performance, especially seed dormancy. The effect of temperature on seed dormancy and flowering time might be mediated by DOG1, seeing the correlation of these traits with DOG1 gene expression and the minimum annual average temperature. Moreover, humanised habitat might be another clue that forced adaptation of seed dormancy and flowering time through DOG1 expression (Chapter 4).

To enhance the knowledge on seed performance, a correlation analysis between seed performance and primary metabolites was performed (Chapter 5). A very strong and positive correlation between germination after artificial aging, which is an estimation for seed longevity, and galactinol content was found in Arabidopsis seeds. This correlation was validated in two crop species: cabbage and tomato. The galactinol content did not change during long-term storage of cabbage seeds, which can be explained by the fact that galactinol accumulates during seed maturation, whereas during seed storage metabolic activity is very low. To understand the role of galactinol in seed longevity, a reverse genetic approach using Arabidopsis T-DNA knock-out lines of genes in the raffinose family oligosaccharides pathway (galactinol synthase 1 (gols1), galactinol synthase 2 (gols2), raffinose synthase (rs5), stachyose synthase (sts) and alpha-galactosidase (agal)) and overexpressors of the cucumber GALACTINOL SYNTHASE 2 gene were used. Galactinol
levels of the _gols2_ mutants and the _gols1gols2_ double mutant were significantly lower than those of wild type Columbia-0 (Col-0) and seeds of these mutants displayed significantly reduced seed longevity when compared to Col-0. The consistent positive correlation between seed galactinol content and seed longevity makes galactinol a potential biomarker for seed longevity.

Finally, I integrate and discuss the work presented in this thesis in **Chapter 6**. The effect of the environment during seed maturation on subsequent seed performance is emphasized, covering the phenotypic plasticity in response to environmental factors and showing the importance of temperature during seed maturation for seed performance. I discuss the influence of environmental changes on _DOG1_ and the fact that _DOG1_ not only plays a role in seed dormancy but also in seed longevity. The identification of galactinol as a potential biomarker of seed longevity is of great importance for the seed industry, since it may facilitate making decisions on which seed lots can be placed onto the market or can be stored for longer periods of time.
Samenvatting

Klimaatveranderingen hebben een enorm effect op planten en kunnen zelfs leiden tot het uitsterven van een soort. Planten beschikken echter over verschillende overlevingsstrategieën om stressvolle condities te overleven, en één van deze strategieën is overleving als zaad. Het bestuderen van de interactie tussen genotype en omgeving heeft geleid tot een beter inzicht in hoe de omgeving zaadeigenschappen kan beïnvloeden. In Hoofdstuk 2 zijn zaden van twaalf Arabidopsis thaliana genotypen, die zich hebben ontwikkeld in verschillende omgevingscondities (standaard condities, lage en hoge lichtintensiteit, korte dag, continue licht, lage en hoge temperatuur, laag en hoog nitraat en laag en hoog fosfaat), gefenotypeerd voor 18 aan plant en zaad gerelateerde eigenschappen. Temperatuur bleek de meest dominante factor voor zowel plant- als zaadeigenschappen, terwijl lichtintensiteit en fotoperiode alleen een effect op planteigenschappen hebben. Voeding, verschillende concentraties nitraat en fosfaat, aangeboden aan de moederplant, hadden het geringste effect op plant- en zaadeigenschappen. Genotype x omgevingsinteracties bleken zeer significant in deze studie. Bijvoorbeeld voor kiemrust, waar de meer dormante genotypen – zoals de bijna isogene lijnen (NILs) van de Delay of Germination (DOG) genen NILDOG1, NILDOG3, NILDOG6 en een ABA-katabolisme mutant (cyp707a1-1) – een interactie lieten zien met laag nitraat en lage temperatuur. Deze condities leidden tot nog hogere kiemrust niveaus in deze genotypen.

Om het effect van de omgeving op zaadeigenschappen te bestuderen hebben we de niet-transgene lijnen uit Hoofdstuk 2 ook in het veld geplant (Hoofdstuk 3). Zaden zijn gezaaid in de zomer, herfst, winter en het voorjaar en de zaadeigenschappen van de nakomelingen van deze planten zijn geanalyseerd. Bodem- en luchttemperatuur en daglengte tijdens de groeiperiode werden geregistreerd. De uitkomsten van dit experiment (Hoofdstuk 3) zijn vergeleken met die van de experimenten onder gecontroleerde condities (Hoofdstuk 2). Bij het korter worden van de daglengte en het lager worden van de temperatuur (zomer experiment) producerden planten zaden die kleiner en dormanter waren. Er waren verschillen tussen de veld- en controle condities. Zaden uit de controle condities waren in het algemeen groter en minder dormant in vergelijking tot alle vier seizoenen in het veld (Hoofdstuk 3). Vergelijking van beide experimenten laat ook zien dat inderdaad de temperatuur tijdens de zaadontwikkelingsperiode voor een belangrijk deel de zaadeigenschappen bepaald, en dan in het bijzonder de kiemrust. Hoewel fotoperiode op zichzelf geen significant effect op zaadeigenschappen had (Hoofdstuk 2), leidt de interactie zoals die voorkomt in het veld (fotoperiode en temperatuur) tot variatie in kiemrust en zaadgrootte (Hoofdstuk 3). In Hoofdstuk 3 heb ik gezien dat kiemrust in grote mate door omgevingsfactoren bepaald wordt, de verschillen veroorzaakt door de omgeving waren groter dan de genetische verschillen tussen de lijnen. Een uitzondering hierop is NILDOG2, van deze lijn is het bekent dat deze wat eerder bloeit dan wildtype Landsberg erecta (Ler) door de aanwezigheid van het CRYPTOCHROME 2 (CRY2), alle van de accessie van de
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Kaapverdische eilanden (Cvi). Dit was ook het enige genotype dat een sterke interactie met de omgeving liet zien. NILDOG2, gezaaid in het voorjaar, produceerde zaden met de geringste kiemrust, gevolgd door respectievelijk zaden geproduceerd in de winter, herfst en zomer.

In Hoofdstuk 4 zijn de aan de fenotypische plasticiteit van kiemrust gerelateerde eigenschappen (zoals kiemrust, kiemsnelheid, zaadgrootte en bloeitijd) en het effect van geografische en omgevingsfactoren in een regionale populatie van 300 Arabidopsis accessies van het Iberisch schiereiland onderzocht. Kiemrust, kiemsnelheid en bloeitijd correleerden met hoogte en latitude. Dit geeft aan dat populaties die geografisch gezien dichter bij elkaar gelegen zijn in het algemeen genetisch gezien meer overeenkomen voor deze eigenschappen. Sterke ‘altitudinale clines’ werden gevonden voor kiemrust, zaadgrootte en bloeitijd, wat inhoudt dat planten van lage altitudes kleinere, meer dormante zaden produceren. Verder zijn kiemrust en zaadgrootte de enige eigenschappen met een significante correlatie met alle klimaateigenschappen, inclusief zonnestraling tijdens de zomer welke het sterkst correleerde met kiemrust. Kiemrust correleerde positief met minimum en maximum temperatuur gedurende het jaar en zonnestraling tijdens de zomer, maar negatief met neerslag tijdens het voorjaar en de zomer. Zadgrootte liet dezelfde patronen zien, maar met tegengestelde effect voor temperatuur, neerslag en zonnestraling. Bloeitijd correleerde negatief met temperatuur en precipitatie tijdens de herfst en winter en niet met zonnestraling. Dit geeft aan dat accessies van warmere en drogere locaties in het Iberische schiereiland naar verwachting eerder bloeien en kleinere zaden produceren met een hogere kiemrust. In Hoofdstuk 2, 3 en 4 laat ik zien hoe belangrijk de omgevingsomstandigheden zijn en dat temperatuur een hoofdrol speelt in de regulatie van zaadeigenschappen, met name voor kiemrust. Mogelijk speelt DOG1 een rol in het effect dat temperatuur heeft op kiemrust en bloeitijd, gezien de correlatie van deze eigenschappen met DOG1 gen expressie. Daarnaast is beïnvloeding van het leefgebied door de mens een andere factor die mogelijk een rol in de adaptatie van kiemrust en bloeitijd via DOG1 expressie speelt (Hoofdstuk 4).

Om meer kennis over zaadeigenschappen te verkrijgen is er een correlatieve analyse tussen zaadeigenschappen en primaire metabolieten uitgevoerd (Hoofdstuk 5). Een sterke positieve correlatie werd gevonden tussen kieming na veroudering, wat een maat is voor zaadlevensduur, en hoeveelheid galactinol in Arabidopsis zaden. Deze correlatie is gevalideerd in twee gewassen: kool en tomaat. Het galactinolgehalte veranderde niet tijdens het langdurig bewaren van koolzaden, wat verklaard kan worden door de lage metabole activiteit in droge zaden. Om de rol die galactinol in zaadbewaarbaarheid speelt verder te onderzoeken is er gebruik gemaakt van Arabidopsis T-DNA mutanten met inserties in genen van de raffinose oligosaccharide pathway; (galactinol synthase 1 (gols1), galactinol synthase 2 (gols2), raffinose synthase (rs5), stachyose synthase (sts) en alpha-galactosidase (agal)) en overexpressie lijnen van het komkommer GALACTINOL SYNTHASE 2 gen. De galactinol gehaltes van de gols2 mutanten en de gols1gols2 dubbel mutant waren
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significant lager dan die van Columbia-0 (Col-0) wildtype en zaden van deze mutanten hadden ook een verminderde levensduur in vergelijking met Col-0. De consistente positieve correlatie tussen galactinolgehaltes en levensduur maken galactinol een potentiële biomarker voor zaadbewaring.

Tenslotte, in Hoofdstuk 6, integreer en bediscussieer ik het werk dat in dit proefschrift gepresenteerd is. De invloed van de omgeving tijdens de zaadrijping op zaadeigenschappen wordt benadrukt. Dit betreft zowel de fenotypische plasticiteit ten opzichte van de omgewingsfactoren als het belang van temperatuur tijdens de rijpingsfase. Ik bespreek het effect van omgewingsfactoren op DOG1 en het feit dat DOG1 niet alleen kiemrust maar ook de bewaarbaarheid van zaden beïnvloed. De identificatie van galactinol als een potentiele biomarker is van groot belang voor de zaadindustrie, omdat dit het mogelijk maakt te bepalen of partijen zaad op de markt gebracht dienen te worden of nog langer bewaard kunnen worden.
Acknowledgements

First of all I am afraid that these acknowledgements will be too long, because I am already working 13 years with seeds and I have to mention a lot of people here. Even though I am sure that I will forget some people (sorry for it, by the way), but over the years my memory is getting pretty bad.

For my first thanks, I need to go back to the year 2002. Back then I had the pleasure to meet Frederico Pereira Ribeiro (master student at that time). He invited me to help him with his thesis, introducing me to the “seed world”. Because of him I started to be a trainee in the Seed Research Laboratory at Federal University of Viçosa (UFV). Thank you so much Fred for showing me how nice it is to work with seeds. I also want to thank Denise C.F.S. Dias for accepting me as a student in her lab and giving me the opportunity to continue my research in seed technology.

I had the opportunity to work with many people during my time at the Seed Research Laboratory at UFV; it is impossible to mention everyone, so thank you all for the support and guidance during these 10 years. However, one researcher I want to thank specifically: Maria Carmen Bhering (Carminha), with whom I had the pleasure to work during this time in Brazil. Thank you for always being there, as a friend and counsellor; thank you for contributing so much during my time at the university; thank you for being part of my private life. I love you so much!

To continue my scientific acknowledgements, I have to give many, many thanks to Henk Hilhorst, my supervisor, first for accepting me as a sandwich-PhD student for one year in the Seed Lab in WUR and especially for attending my defence at the end of my first PhD in Brazil. I was very happy with it. Second, for putting trust in my work and for inviting me to come back to Wageningen to do a second PhD (yes, sometimes I also think that I was crazy to do the second PhD, but now I am sure that this was a very good decision; I grew so much in the my scientific and personal life during the last 3 years). Henk, thank you for welcoming and guiding me in the research group and also for providing a stimulating and good working environment.

During my first year in Wageningen I had the opportunity to be guided by two very special persons that taught me a lot of things: the big man Wilco Ligterink and the sweet Hanze He. Wilco, thank you for your patience, input and especially for showing me how things work in the lab. And my dear Hanzi, I have no words to express the gratitude for you. Without you, I would never be here today. It was a pleasure to divide the project with you and to always work together. I am sure that our friendship will last forever.

I need to admit that in the begging of my first year in Wageningen I was a little bit scared of two colleagues, but after getting to know them better, I discovered that they are THE BEST. The first person is Leo Willems. Leo thanks for being always very efficient and for being always available to help me, you are the best technician ever. Thank you also for becoming my friend and for introducing me to your lovely family. I hope we will keep
in touch with each other. I am looking forward to have fun with the kids. The other person that I would like to express my most sincere gratitude too is the best supervisor ever, Leónie Bentsink. Leónie, without your support and supervision I would not be here today. Thank you for teaching me the value of science. And that it is possible to be successful in your work and to be a very good mother at same time. Thank you for the countless talks about work and personal life, and especially for the huge amount of “paper tissue” that you always prepared for me. It was very helpful in though meetings. Thank you for everything Leónie and Leo.

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Back to the Seed Lab I would like to thank all the colleagues that I had the great opportunity to work with during these 4 years of my PhD. Thank you for your friendship and all the fun that we had together. It really was a very good and unforgettable time. But in particular I need to thank you, Julio (titio). Without your support during my first days in Wageningen I would have returned to Brazil on my first day. I still don’t know if I would have managed to arrive in Wageningen (with my 2 “small” suitcases of 32 kg + 2 hand bags) alone. Also to register in the municipally, bank and of course make a lot of new friends, without you, my friend, I would be nothing in the Netherlands. Also thanks a lot Alexandre (Xande) for your help. Even though your slow motion style sometimes makes me crazy, I would not be able to perform very big experiments like the “Iberian Population” without you. I also would like to thank the people already left our group: Cimille (Baiana), Leticia, José Contado, Cihan Tas, Juliana, Gabriela, Manuela, Somayeh, Ronny, Rashid, Noorullah, Marieke, Cristiane Carvalho, Phuong, Wilson, Cristiane Brito, Rafael, Ludymila, Kerstin, Ezequiel, Thiago, Paulo Ribeiro and the several BSc and MSc students that were part of this amazing group. And of course I would like to thank my colleagues that are still part of this group: Maria Cecília, Mariana, Natalia Moreno, Bas, Harm, Bing, Gonda, Elise, Anderson, Farzaneh, Nafiseh, Juriaan, Richard Immink, Melissa, Lukasz, Paulo de Carvalho, Hiro and Mariko Nonogaki. Thank you, thank you and thank you.

I also have very great memories about all members of the Plant Physiology department. We had nice work discussions, but also nice lunches, trips, dinners and parties together. I should not forget the secretaries: Rina and Margaret; the technicians: Jacqueline, Diaan, Marielle and Francel. Staff members: Dick Vreugdenhil, Sander van der Krol and Robert Hall; the post docs: Emilie, Kristyna, Iris, Carolien, Lidia, Thierry and Yanxia; and of course the great PhD friends: Beatriz, Arman, Xi, Jun, Mark, Esmer, Karen, Sang, Giovanni, Umidjon, Mahdere, Manus, Bo, Yuanyuan, Yunmeng; and other friends of the plant physiology that are now doctors: Natalia Carreno, Desalegn, Benyamin, Neli, Ralph,
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Carlos Alonso-Blanco and Xavier Picó: thank you for the collaboration. Both of you were essential pieces to make one chapter come true. Thank you for the countless emails, skype meetings, analysis of the data, support and shared knowledge that you have with me.

My life in Wageningen does not only consist of friends from university, and I would like to mention a couple of special friends here. My Brazilian friends from Wageningen: Felipe, it is for your “fault” that I met Joachim, thank you again for it. Anabele, Wiebe, Mateus & Eduardo, thanks for all the barbecues, Sinterklaas and Christmas evenings in your house. You really make me feel better at special times such as at Christmas, where I was away from my family. Fernanda Paganelli & Mauricio, Fernanda Smiderle, Carol & Denis, Ana Carolina & William, Cinara & Janio, Saulo & Suzanne, Thiago Okubo, Zany, Rafinha, Mina, Alvaro, Denis Santiago (my favourite singer), Flavinha, Charles, Ulisses, Ana Paula, Greice. Ex housemates: Daniel and Daniela, Abhishek, Alexandra, Willemijn and Ioanna. The “latino” friends: Ana Marcela & Rafael, Carlos, Irene, Sergio & Madelaine, Paola, Claudio, Valeria. And some others that I forgot to mention here: thanks all to shared unforgettable moments with me. I hope we can continue to keep in touch and share more and more moments together.

I would like to thank my paranymphs Renake and Carla. Renake is not only part of the Seed Lab but also a very good friend outside of the university. Rê thanks for the moral support, care, favours, and affection not only for me but also for my son. And Carla, you are the best neighbour in the world. I miss you so much already. Thank you for organising Oliver’s baby shower, my “weeding party” and so many other things.

Now I have a new family in Europe, actually in Germany. Hannelore, Dietrich, Vinzenz and Paulina thanks for accepting me to be part of your family. Thank you for all support during these years.

I also want to thank all my friends and family in Brazil, it is impossible to mention names here, otherwise I would have to write the same amount of pages as my entire thesis. But I need to thank especially my mother Zeni, my father José Ulisses, my sister Isabella and my lovely nephew Bernardo for supporting all my decisions and always being present in my life, even from the distance. You are my safe harbour. Eu amo muito vocês!

Last but not least, thank you my beloved husband Joachim for all your patience, help in difficult times and for all the emotional support, care and most importantly for our son Oliver Lucas, who changed our life forever, but to a very good side. Ich liebe dich! Vocês são minha vida!

Furthermore, thanks to all that somehow contributed to this work and who believed in me.
Deborah de Souza Vidigal was born on 15 December 1980 in Ubá, Minas Gerais, Brazil. In 2001 she joined the undergraduate program in Agronomy at the Federal University of Viçosa (UFV). During her bachelor studies, she followed a trainee program in the Seed Research Laboratory at UFV, working with seed vigour during maturation, seed longevity and osmopriming. After her graduation in 2006, she started her MSc in the department of Crop Science at the same university. In her thesis she investigated the effect of physiological and biochemical alterations in pepper seeds during fruit maturation and post-harvest storage of the fruits. She continued her research as a PhD in the same department in 2008. As part of an exchange program, she went as sandwich-PhD to the Seed Lab at the Wageningen UR, Netherlands. After returning to Brazil 2012, she defended her thesis on the “Effect of the parental environment on Arabidopsis thaliana seed quality”, focusing on the impact of maternal phosphate levels on seed quality, and seed dormancy in particular. At the end of her first PhD, Dr. Henk W.M. Hilhorst invited her to return to the Netherlands to do a second PhD at the Wageningen UR under his and Dr. Leónie Bentsink's supervision. She continued to work on the same topic as her previous PhD, namely “Production environment and seed quality”. The results of this project are described in this thesis.
Publications


# Education Statement of the Graduate School

## Experimental Plant Sciences

**Issued to:** Deborah de Souza Vidigal  
**Date:** 14 December 2015  
**Group:** Laboratory of Plant Physiology  
**University:** Wageningen University & Research Centre

## 1) Start-up phase

- **First presentation of your project**  
  Influence of maternal growth conditions on seed quality  
  Oct 10, 2011
- **Writing or rewriting a project proposal**
- **MSc courses**
- **Laboratory use of isotopes**

**Subtotal Start-up Phase** 1.5 credits*

## 2) Scientific Exposure

- **EPS PhD student days**
  - EPS PhD student day, Utrecht University, Utrecht, NL  
    Jun 01, 2010  
  - EPS PhD student day, Leiden University, Leiden, NL  
    Nov 29, 2013
- **EPS theme symposia**
  - EPS theme 1 'Developmental Biology of Plants', Leiden University  
    Jan 20, 2011  
  - EPS theme 3 'Metabolism and Adaptation', Wageningen University  
    Feb 10, 2011  
  - EPS theme 3 'Metabolism and Adaptation', University of Amsterdam  
    Mar 22, 2013  
  - EPS theme 3 'Metabolism and Adaptation', Wageningen University  
    Mar 11, 2014
- **NWO Lunteren days and other National Platforms**
  - ALW meeting 'Experimental Plan Sciences', Lunteren  
    Apr 19-20, 2010  
  - ALW meeting 'Experimental Plan Sciences', Lunteren  
    Apr 22-23, 2013  
  - ALW meeting 'Experimental Plan Sciences', Lunteren  
    Apr 14-15, 2014  
  - ALW meeting 'Experimental Plan Sciences', Lunteren  
    Apr 13-14, 2015
- **Seminars (series), workshops and symposia**
  - Invited seminar Christian Hermans, 'Molecular basis of plant nutrition: Insights into the responses to magnesium and nitrate availability'  
    Dec 01, 2010  
  - Invited seminar Steven Penfield (University of Exeter, Exeter, UK):  
    'Parenting in plants: maternal control of seed dormancy'  
    Jun 12, 2012
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<td>Invited seminar Jill M. Farrant (University of Cape-Town, South Africa): 'Use of resurrection plants as model to understand how plants tolerate extreme water loss: a systems biology approach with applications for making drought tolerant crops'</td>
<td>Jun 26, 2012</td>
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<td>1st Dutch Seed Symposium 'Improving seed quality'</td>
<td>Oct 02, 2012</td>
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<td>Invited seminar Ruth Finkelstein (University of California, Santa Barbara, USA): 'ABA signaling network in Arabidopsis'</td>
<td>Nov 14, 2012</td>
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<td>Invited seminar Aaron Fait (Ben-Gurin University of Negev, Ben-Gurion, Israel): 'Tackling natural variance in seed metabolism integrating metabolite profile via network analysis'</td>
<td>Dec 04, 2012</td>
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<td>Invited seminar Detlef Weigel (Max Planck Institute for Developmental Biology, Tübingen, Germany): 'Origin and consequences of genetic and epigenetic variation in Arabidopsis thaliana'</td>
<td>Feb 02, 2013</td>
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<td>Plant Science Seminar on Bioinformatics - (Gabino Sanchez Perez, Bioinformatics at PRI, Wageningen University): 'Is your research becoming Digital? Time to call the BioinforMagician!'</td>
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<td>Invited seminar Marc Boutry (University de Louvain, Belgium): 'Plant drug smugglers'</td>
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<td>Public lecture Plant metabolomics by Robert Hall: 'The potential of Plant Metabolomics'</td>
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<td>CBSG seminar Kazuki Saito (RIKEN Plant Science Centre and Chiba University, Japan): 'Metabolomics-based functional genomics - from Arabidopsis to crops and medicinal plants'</td>
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<td>Farewell and Mini-Symposium Prof. Maarten Koornneef: 'Arabidopsis in Wageningen'</td>
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<td>KLV workshop - “Scientific English, avoiding non-native influences”</td>
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<td>Seminar plus</td>
<td>3rd Dutch Seed Symposium</td>
<td>Oct 7, 2014</td>
</tr>
<tr>
<td>International symposia and congresses</td>
<td>3rd ISSS workshop on Molecular Aspects of Seed Dormancy and Germination - York, UK</td>
<td>Jul 18-21, 2010</td>
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<td></td>
<td>10th ISSS Conference of the International Society for Seed Science - Brazil</td>
<td>Apr 10-15, 2011</td>
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<td></td>
<td>4th ISSS workshop on Molecular Aspects of Seed Dormancy and Germination - Paris, Fr</td>
<td>Jul 9-12, 2013</td>
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<tr>
<td></td>
<td>ISSS workshop on Seed Longevity - Wernigerode, Germany</td>
<td>Jul 5-8, 2015</td>
</tr>
<tr>
<td>Presentations</td>
<td>Poster presentation</td>
<td>10th ISSS Conference of the International Society for Seed Science, Brazil</td>
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<td></td>
<td></td>
<td>4th ISSS workshop on Molecular Aspects of Seed Dormancy and Germination, Paris, Fr</td>
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<td></td>
<td>ALW meeting 'Experimental Plan Sciences', Lunteren</td>
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<td></td>
<td></td>
<td>ISSS workshop on Seed Longevity, Wernigerode, Germany</td>
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<tr>
<td>Oral presentation</td>
<td>10th ISSS Conference of the International Society for Seed Science, Brazil</td>
<td>Apr 10-15, 2011</td>
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<td></td>
<td>ALW meeting 'Experimental Plan Sciences', Lunteren, NL</td>
<td>Apr 13-14, 2015</td>
</tr>
<tr>
<td>IAB interview</td>
<td>Meeting with a member of the International Advisory Board (IAB) of EPS</td>
<td>Nov 14, 2012</td>
</tr>
<tr>
<td>Excursions</td>
<td>Rijk Zwaan (visit seed and breeding company), de Lier</td>
<td>Sep 27, 2013</td>
</tr>
</tbody>
</table>

**Subtotal Scientific Exposure:** 19.5 credits*
3) In-Depth Studies

► EPS courses or other PhD courses
  Master Class Seed Technology - Wageningen       May 21-24, 2012
  System biology: Statistical analysis for -omics data Dec 10-14, 2012

► Journal club
  Literature discussion group at Plant Physiology, WUR 2012-2015

► Individual research training

Subtotal In-Depth Studies 7.2 credits*

4) Personal development

► Skill training courses
  Information Literacy including EndNote Introduction Oct 30-31, 2012
  Techniques for Writing and Presenting a Scientific Paper Apr 23-26, 2013
  Summer course English for IELTS Aug 19-30, 2013
  Project and Time Management Sep 17, Oct 01 & 29, 2013
  Competence Assessments Sep 10, Oct 09, 2013

► Organisation of PhD students day, course or conference

► Membership of Board, Committee or PhD council
  Activities Committee Plant Physiology 2013

Subtotal Personal Development 5.9 credits*

TOTAL NUMBER OF CREDIT POINTS* 34.1

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

* A credit represents a normative study load of 28 hours of study.
This work was performed at the Laboratory of Plant Physiology, Wageningen University and Research Centre, with financial support from the Dutch Technology Foundation STW, which is part of the Netherlands Organisation for Scientific Research (NWO) and partly funded by the Ministry of Economic Affairs (project numbers STW 11314 and STW 12951)

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