The genetics of seed quality in *Arabidopsis thaliana*

De genetica van zaadkwaliteit in *Arabidopsis thaliana*

Promotor:	Prof. dr. ir. M. Koornneef Persoonlijk hoogleraar bij het laboratorium voor Erfelijkheidsleer, Wageningen Universiteit
Co-promotor	dr. S.P.C. Groot Clusterleider bij Plant Research International, Wageningen University and Research Centre Coördinator Wageningen Seed Centre
Promotiecommissie	Prof dr. D. Bartels, Universität Bonn, Duitsland dr. ir. F. A. Hoekstra, Wageningen Universiteit Prof. dr. L.H.W. van der Plas, Wageningen Universiteit Prof dr. ir. P. Stam, Wageningen Universiteit

Emile J.M. Clerkx

The genetics of seed quality in *Arabidopsis thaliana*

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. dr. ir. L. Speelman
in het openbaar te verdedigen
op maandag 19 januari 2004
des namiddags te vier uur in de Aula

ISBN 90-5808-970-3

Contents

Chapter 1

General introduction

Introduction

Surviving unfavorable growth conditions, like cold or drought, is essential for sessile plant species. One way for plants to survive such conditions is by their seeds. Orthodox seeds can survive in a desiccated state and often exhibit dormancy or metabolic quiescence (Farnsworth, 2000), in which they can remain viable for a long time. There are indications of seeds keeping their viability for 1000 years, although the evidence about their age is circumstantial. Records, where the seed age can be verified, show that some species can remain viable for 200 years (Bewley and Black, 1994), although none of these seed were stored at low temperature and humidity, which would normally enhance their storability even more. Hay et al. (2003) predicted that the longevity for Arabidopsis seeds could be approximately 2000 years if stored at –20°C and at 5% moisture content.

Seed quality

Dickson (1980) defined high quality seeds as undamaged seeds that have a high level of germination, which will produce uniform, vigorous seedlings without defects under various environmental conditions. The maintenance of seed quality can be seen as the ability to withstand stresses that occur when exposed to unfavorable conditions during germination or while stored. Seed deterioration can already start during growth of the plant where pathogens could render it physiologically ill equipped to produce good quality seeds (McGee, 2000). Unfavorable environmental conditions can have a similar effect. Early sprouting, in extreme cases vivipary, involving the absence of primary dormancy in combination with wet weather during seed ripening and at harvest time, causes losses in seed quality as well (Coolbear, 1995). During harvest and subsequent handling seeds could sustain mechanical damage, which is different from physiological damage inflicted during storage (McDonald, 1999).

Damage sustained during storage could be the result of seed-borne bacteria and fungi, carried over from the production field. During seed storage bacterial growth is usually no real problem since bacteria are unable to grow on substrates with relatively low moisture contents, like dry seeds. However fungi are able to grow over a wide range of temperature and relative humidity (RH), decreasing the seed quality as they grow (Kulik, 1995). Seeds deteriorate physiologically also in the absence of pathogens. Seed deterioration during seed aging is probably best described as the loss of seed quality with time (Coolbear, 1995). Seed longevity is affected by various environmental factors like RH, and the seed moisture content resulting thereof, and storage temperature. At a given temperature, there is a logarithmic relationship between the longevity of orthodox seeds, which are desiccation tolerant, and

their storage moisture content. This indicates that preservation of orthodox seeds is relatively easy when low moisture content and low temperature are maintained. However, there is a limit to the moisture content, since lowering the moisture content of stored seeds below a species-specific threshold, will not increase longevity any more (Ellis et al. 1990) and might even result in desiccation damage. In mature dry orthodox seeds, usually water only exists as bound water, which is virtually immobile and is sufficiently structured so that thermodynamic properties differ from those of free bulk water. Most significantly, it is not readily freezable, and hence many dry seeds can withstand subzero temperatures (Bewley and Black, 1994).

Seed quality also includes uniform germination and crop establishment, which can be enhanced by seed priming. Seed priming usually involves re-hydration of seeds but preventing radicle protrusion. Handling and long-term storage of primed seeds requires drying the primed seeds to a low moisture content. Conflicting results regarding primed seeds have been obtained since both reduced and equal longevity compared to non-primed seeds have been reported (Pill, 1995; McDonald, 1999 and references therein).

All organs that make-up the seed can sustain physiological damage: the seed coat, which is of maternal origin and which acts as a physical and chemical barrier, the embryo, the endosperm and the cotyledons. Reduction in seed vigor has been related to various seed properties such as color, weight and membrane composition, although the correlation between reduced seed quality and such traits is often species, or in some cases even variety, specific (Dickson, 1980; McDonald, 1999).

The development and mechanism of desiccation tolerance in seeds

Tolerance to desiccation is a survival mechanism adopted by orthodox seeds and allows seeds to survive under extreme environmental conditions and favors wide dispersal (Wilson Jr, 1995; Ingram and Bartels, 1996). Furthermore it allows these seeds to be stored. Desiccation tolerance in seeds develops during seed maturation, after morphogenesis of the embryo is completed (Koornneef et al., 1989; Bewley and Black 1994; Ingram and Bartels, 1996). During the last period of seed maturation, well beyond the moment desiccation tolerance has been acquired, seeds gain in longevity (Hay et al., 1997; Jalink et al., 1998). In orthodox seeds desiccation tolerance is associated with the ability to avoid protein unfolding, restriction of membrane disturbance and curtailing the production of reactive oxygen species (ROS). In desiccation tolerance it is often found that one mechanism does not confer tolerance on its own, but the interplay of several mechanisms simultaneously is essential (Hoekstra et al., 2001). Upon water loss the cellular volume decreases, causing crowding of cytoplasmatic components, increasing the chance of molecular interactions that can cause

protein denaturation and membrane fusion (Hoekstra et al., 2001). Desiccation induced membrane damage can be measured as increased conductivity upon rehydration of desiccated seed tissues. The rate and extent of cytoplasmatic leakage is positively correlated with the degree of desiccation sensitivity (Leprince et al. 1993). Protection of proteins and membranes during moderate desiccation occurs by water replacement. Water molecules are replaced by sugars at the hydrogen bonding sites to preserve the native structure of proteins and the spacing between phospholipids (Hoekstra et al., 2001). Besides sugars heat inducible hydrophilic late embryogenic abundant (LEA) proteins can act as molecular chaperones (Hoekstra et al., 2001). Bettey and Finch-Savage (1998) investigated the role of two subclasses of LEA's in *Brassica* seeds, dehydrins and small heat shock proteins (sHSP). The content of dehydrins did not show a positive relation with seed performance, however the sHSP17.6 content did show a positive correlation (Bettey and Finch-Savage, 1998). Another clue to a role for sHSP's could come from the fact that in Arabidopsis cytosolic sHSP's appear to respond to specific developmental signals associated with the acquisition of desiccation tolerance (Wehmeyer and Vierling, 2000). During further dehydration a biological glass, an amorphous metastable state that resembles a solid, brittle material, but with retention of the disorder and physical properties of a liquid, is formed (Hoekstra et al., 2001). In dehydrating seeds, soluble carbohydrates form a glassy state in which cellular membranes and proteins are likely to be protected; furthermore, macromolecular mobility is decreased. Oligosaccharides, such as raffinose and stachyose, are more effective glass formers than sucrose or mono-saccharides, the latter having a plasticizing effect. The absence of mono-saccharides in dry desiccation tolerant seeds gives support to the importance of a solid-like glassy matrix. Proteins tend to stabilize the glass and here again LEA's might play a role in forming a dense stable glass (Wolkers et al., 1998). Besides the membrane and protein stabilizing function of the glassy matrix, the high viscosity decreases molecular mobility and impedes diffusion within the cytoplasm, thus slowing deleterious reactions and changes in structure and chemical composition during aging. Molecular mobility is increasingly considered to be a key factor influencing storage stability, because it is thought to control the rate of detrimental aging reactions responsible for reducing longevity (Buitink et al., 2000). Recalcitrant seeds are in contrast to orthodox seeds, desiccation intolerant and are found in a wide range of plant species (Farnsworth, 2000). Maintenance of these seeds in seed banks, poses a great challenge to seed physiologists. The moisture content in these seeds should not drop below a relatively high value, between 12 and 31% moisture content, otherwise viability is lost (Chin, 1995). However, even at these conditions maintaining viability does pose problems. In recalcitrant seeds cryopreservation of embryonic axis can be used to preserve germplasm (Wesley-Smith et al., 2001).

The mechanisms of damage to dry seeds after storage

One of the detrimental aging reactions most likely involved in seed deterioration during storage is the production of ROS (Hendry, 1993). ROS are generated in both stressed and unstressed cells, and at various cell compartments. In seeds the most likely source of ROS are the peroxysomes, the mitochondria, autoxidation in the cytosol and through chlorophyll, which is normally degraded during maturation. ROS could directly, or via subsequent lipid peroxidation, lead to mitochondrial dysfunction, enzyme inactivation, membrane perturbation and genetic damage (Coolbear, 1995). In addition to the amount of ROS generated, the final damage is also determined by how efficiently the cells, in which these reactive molecules are formed, are able to scavenge and dispose of them.

The antioxidant defense system in plants consists of both non-enzymatic and enzymatic antioxidants. The latter are only functional when sufficient water is available. In dry seeds only molecular, non-enzymatic, antioxidants (e.g. glutathione, ascorbate, polyols, carbohydrates, peroxyredoxin, tocopherol and phenolics) can alleviate oxidative stress (Hoekstra et al., 2001). Superoxide dismutase (SOD) (Kliebenstein et al., 1998; Grene, 2002) catalyzes the conversion of superoxide radical to H_2O_2. Hydrogen peroxide can be disposed of by catalase and ascorbate peroxidase. Catalases convert H_2O_2 to water and oxygen. Ascorbate peroxidase forms water and dehydroascorbate from ascorbic acid (vitamin C) and H_2O_2 (Willekens et al., 1995; Noctor and Foyer, 1998; Blokhina et al., 2003). Furthermore H_2O_2 removal via ascorbate peroxidase requires glutathione since ascorbate is reduced by dehydroascorbate reductase using glutathione (GSH) as a reducing substrate, which is known as the ascorbate-glutathione cycle (Noctor and Foyer, 1998). Antioxidants such as vitamin E (α-tocopherol) act against phospholipid radicals (Grene, 2002). Other antioxidants are phenolic compounds, among them flavonoids that are abundant in plant tissues (reviewed by Grace and Logan, 2000). Peroxiredoxins, classified as “atypical LEA’s”, have been isolated and characterized as antioxidants from many organisms and their expression in both barley and Arabidopsis is restricted to seeds and their expression is during mid and late seed development (Haslekas et al., 1998; Stacy et al., 1999).

All these processes have been extensively analyzed in plant tissues. The study of these processes in seeds is complex, especially when they are in a strongly desiccated state. Adding water to dry seeds could start germination processes, which will subsequently activate the antioxidant defense system. In a large number of publications correlative evidence is provided that both the amount of oxidants and the antioxidant activity correlates with seed quality (Senaratna et al., 1988; Puntarulo et al., 1991; Cakmak et al., 1993; Leprince et al., 1994; Bailly et al. 1996; Tommasi et al., 2001). Another indication for the importance of free radical scavenging to prevent seed aging could come from recalcitrant

seeds. These contain higher amounts of ascorbic acid compared to orthodox seeds. This might indicate that moist recalcitrant seeds need a constant high level of protection against ROS (Tommasi et al., 1999).

The role of seed dormancy

Dormancy is defined as the failure of an intact, viable seed to germinate under favorable conditions (Bewley, 1997). Primary dormancy develops in seeds while attached to the plant and can be due to a restraint of the seed coat (coat enhanced dormancy) or due to a restricted growth potential of the embryo (embryo enhanced dormancy). The process of embryo dormancy has been studied using mutants defective in seed development and hormonal regulation. One of the hormones regulating dormancy, or rather promoting germination, is gibberellin (GA). GA deficient mutants are unable to germinate without exogenous GA. Furthermore de-novo biosynthesis is required during imbibition since inhibitors of GA biosynthesis prevent germination unless ABA is absent. The important role of abscisic acid (ABA) in seed dormancy and germination was firmly established by the analysis of ABA insensitive and biosynthesis mutants. The absence of ABA-induced dormancy allows seeds to germinate without gibberellins; furthermore maintenance of dormancy is an active process and requires correct ABA signaling (Bentsink and Koornneef, 2002). Hilhorst (1998) suggested that dormancy was associated with the degree of fatty acid saturation; pre-chilling may result in higher levels of unsaturated fatty acids, which could be more susceptible to free radical attack. This would imply that dormant seeds are less sensitive to free radical attack and hence have a better storability than non-dormant seeds (Hilhorst, 1998). It is not clear if dormant seeds, that might be metabolically more quiescent than non-dormant seeds, have an enhanced longevity. Quantitative trait loci (QTL) analysis in the Landsberg *erecta* (L*er*)/Cape Verde Islands (Cvi) recombinant inbred line (RIL) population did not provide evidence of a common genetic control of seed longevity and dormancy (Bentsink et al., 2000; Alonso-Blanco et al. 2003).

Seed that have been dispersed and lost their primary dormancy can induce secondary dormancy in response to unfavorable germination conditions. Anaerobic conditions, darkness or far-red light, temperatures above and below optimal germination temperatures and water stress can induce secondary dormancy. The mechanism of induction is unknown but inhibitors have been invoked, though there is no evidence that ABA is involved. Indeed, even seeds of ABA deficient mutants of Arabidopsis can enter secondary dormancy in response to high temperature (Bewley and Black, 1994).

Techniques for monitoring seed quality loss

Probably the most obvious way to test the physiological and genetic basis of seed longevity is by storing seeds under favorable conditions at low temperature and humidity. Thereafter, their viability and vigor have to be tested by withdrawal of seeds at regular intervals. Then determination of the germination percentage and seedling quality has to be correlated to physiological and genetic properties. However, since under favorable storage conditions orthodox seeds can remain viable for many years, it might be attractive to study mutants with a reduced storability such as those defective in maturation as discussed hereafter.

A faster procedure that could be used to compare seed lots of orthodox species, is the use of accelerated aging, usually called controlled deterioration. Controlled deterioration tests (CDT) simulate aging of seeds under controlled conditions and can be used to predict seed storage potential (Hampton and TeKrony, 1995). This involves the storage of seeds for a short period (hours/days) at high RH (80 to 100%) and temperature (above 40°C). For most commercial crops these conditions are listed in the AOSA Seed Vigour Testing Handbook (AOSA, 1983). Survival can thereafter be quantified as either the number of days CD treatment required to reach 50% germination (D_{50}) or as the total number of germinated seeds at each day of treatment. In addition the number of abnormal seedlings, the nature of abnormalities and the speed of germination could be indicative for seed vigor. These properties can also be assessed after storage at slightly elevated RH (60%). However, this procedure then requires a longer storage period before differences can be observed (Clerkx et al., 2003). Seed deterioration during CDT is often correlated with the accumulation of free radicals (Gidrol et al., 1989; Khan et al., 1996; Bailly et al., 1997).

Viability loss during dry storage of seeds could be due to damage to membranes. Membrane damage results in leakage of electrolytes. Therefore the amount of electrolytes released from imbibing seeds and measured as conductivity, can be used as a method to assess quality of seeds (Illipronti et al., 1997). Increasing conductivity has been linked to decreasing viability. However there are instances where conductivity has not been proven useful in assessing seed quality (McDonald, 1998). Seed vigor is also tested by germination of seeds under adverse conditions like heat, cold and drought stress (Foolad et al. 1999; Bettey et al. 2000). Furthermore *in situ* Fourier transform infrared microscopy has been used to study the heat stability of proteins and properties of the glassy matrix in dry mature seeds, which in turn might be linked to the success of survival in the desiccated state (Wolkers et al., 1998).

The genetics of seed quality

Very little is known about the genetic basis of differences in seed quality because this trait is affected strongly by environmental factors, during seed formation, harvest and storage. This can be illustrated by genetically identical seed lots in which individual seeds, even when grown under identical conditions or even when coming from the same plant, may loose their viability at different intervals after harvest. Genetic studies require differences within the same species. The most obvious difference in longevity can be found between orthodox and recalcitrant seeds. Both orthodox and recalcitrant seeds can be found within the genus *Acer* (Greggains et al., 2000), although a tree species is not very attractive for genetic research. Among orthodox seeds differences are found as well. One of the earliest reports on the genetics of seed quality is by Lindstrom (1942), who found differences in seed longevity in different F_1 hybrids of maize. Genetic differences for viability in open storage can be observed in maize varieties where hard flint and dent varieties remain viable longer then starchy or sweet varieties. However, in closed storage, at fairly constant moisture contents, few differences are evident (Bewley and Black, 1994). A more comprehensive report on the genetics of seed quality is by Dickson (1980). He concluded, as did Lindstrom (1942), that many of the traits influencing seed vigor are of quantitative genetic nature. Studying quantitative traits has been more difficult than monogenic inherited traits. This was until the use of molecular markers for the development of linkage maps allowed one to associate specific regions on a chromosome with the genes controlling the variation for this trait between both parents. These maps provided the foundation for the modern-day quantitative trait loci (QTL) mapping methodologies (Doerge, 2001).

Only recently some QTL studies on seed vigor and longevity have been initiated in crop plants. Bettey et al. (2000), identified in cabbage (*Brassica*) several loci involved in seed vigor by applying heat en water-stress during germination. Furthermore they measured leakage/membrane integrity. However, this trait could not be correlated with seed performance. In tomato (*Lycopersicon*) Foolad et al. (1999) identified QTLs specifically for germination under cold and salt stress. Seed vigor QTL analysis in rice (*Oryza sativa*; Cui et al., 2002) identified several genomic regions controlling germination rate and some of them co-located with seed properties like seed weight and reducing sugar content. Miura et al. (2002), determined seed longevity in rice using a controlled deterioration assay and detected three QTLs. Two different barley (*Hordeum vulgare*) populations were used by Mano and Takeda (1997) to identify loci involved in germination under saline conditions, a stress condition used to determine seed vigor. Cold germination in *Sorgum bicolor* revealed two QTLs influencing this trait (Natoli et al., 2002).

The genetics of seed quality in *Arabidopsis thaliana*

Arabidopsis is often used for genetic analysis because of its many advantages in combining genetics, physiology and molecular biology that have made it a major model system for plant biology (Meinke et al., 1998). *Arabidopsis thaliana* is a small weed that belongs to the mustard family (Brassicaceae) and has a broad distribution throughout the Northern Hemisphere (Hoffman, 2002). Many different accessions have been collected from natural populations; together with many induced mutants these are available for experimental analysis. The advantages of using Arabidopsis for genetic research are that this small self-fertilizing species has a short life cycle of 6 to 8 weeks and has a small genome of 130 megabase, divided over 5 chromosomes. This genome is completely sequenced (AGI, 2000) and contains approximately 26000 genes and provides, together with an almost complete collection of knock-out mutants, a major resource for molecular research (http://www.arabidopsis.org).

The genetics of seed quality in *Arabidopsis thaliana*: existing mutants

The role of antioxidants can either be investigated physiologically, by correlating the antioxidant status to the loss of vigor, or by using mutants affected in these processes. For instance the *frostbite1* (*fro1*) mutant described recently by Lee et al. (2002) was shown to accumulate ROS constitutively in leaves. To further investigate the role of antioxidants a set of previously described mutants with reduced antioxidant levels can be used. These are the vitamin C (L-ascorbic acid) deficient mutant (*vtc1-1*; Conklin et al. 1996), a glutathione deficient mutant (*cad2-1*; Howden et al., 1995) and mutants defective in their catalase function (Salomé and McClung, 2002).

The seed coat, besides its function in protection against mechanical damage, is also a physical barrier for water and gases. In case of oxygen, a dense impermeable seed coat could hinder oxygen uptake slowing down metabolism and possibly ROS formation. For two Arabidopsis seed coat mutations a reduction in seed vigor has been described before (Debeaujon et al., 2000). One of them is the *transparent testa4* mutant (*tt4-1*), which lacks chalcone synthase activity (Feinbaum and Ausubel, 1988) and therefore does not accumulate brown tannins in the seed coat. The second seed coat mutant is the *aberrant testa shape* (*ats*) mutant missing two of five integument layers, which could make the seed coat more permeable to various compounds, which might include oxygen (Léon-Kloosterziel et al., 1994).

Genetic differences regarding seed-longevity have been reported in Arabidopsis for seed maturation defective mutants. Mutations in genes like *LEAFY COTYLEDON1* and *2*

(*LEC1*, *LEC2*), *FUSCA3* (*FUS3*) and *ABSCISIC ACID INSENSITIVE3* (*ABI3*) that regulate seed maturation are characterized, among others, by very reduced storability of their seeds (Holdsworth et al., 1999; Finkelstein et al., 2002). The function of these genes is not fully overlapping as is inferred from their mutant phenotype, which especially distinguished the *LEC/FUS3* group from *ABI3*. However, differences between the *LEC1* and *FUS3* genes have also been described (Raz et al., 2000). Cloning of *LEC1* showed it to encode a transcription factor resembling a HAP3 sub unit of a CCAAT box-binding protein (Lotan et al., 1998). *LEC1* acts in concert with other genes like *ABI3*, *LEC2* and *FUS3* (Parcy et al., 1997), which encode B3 proteins. Null alleles of *ABI3* have a reduced expression of many seed maturation specific mRNAs and proteins, like LEA's, and remain non-dormant (Ooms et al., 1993; Parcy et al., 1994). Different, compared to the *lec* and *fus3* mutant seeds, is the fact that *abi3* mutant seeds also display a reduced chlorophyll breakdown at the end of seed maturation, resulting in the typical green seed phenotype (Ooms et al., 1993; Nambara et al., 1995; Parcy et al., 1997) and that they are resistant to the germination inhibiting effects of abscisic acid (ABA). An allelic series of *abi3* mutants has the following order of maturation defectiveness *abi3-5* > *abi3-7* > *abi3-1* > wild-type (Bies-Ethève et al., 1999). This order was found in their tolerance to ABA and also when studying protein stability where wild-type shows the highest stability and *abi3-5* the lowest (Wolkers et al., 1998). In addition to these major regulators of seed maturation, other genes, which may include the target genes of the regulatory genes described above, should affect the development of longevity.

The genetics of seed quality in *Arabidopsis thaliana*: new variation

Studying complex traits, like seed storability and desiccation tolerance by mutagenesis is complicated by the fact that several processes contribute to these traits, each with their own genetic basis. In addition gene redundancy is relatively frequent in Arabidopsis (AGI, 2000). Both aspects make that often only a combination of mutations will reveal the role of these genes in a particular process. Single mutants might have none or very subtle effects but may show a much more pronounced phenotype when they occur in a 'sensitized' genetic background (Matin and Nadeau, 2001). A "sensitized" background has a mutation in the process under study, which allows the identification of so-called enhancer and suppressor mutants (Page and Grossniklaus, 2002). Effects of the genetic background on the expression of specific alleles of genes are often described as modifiers (Nadeau 2001). Depending on the nature of the phenotypic effect, modifiers might cause more extreme (enhanced) phenotypes, less extreme (reduced, or even suppressed) phenotypes or novel (synthetic) phenotypes. Such modifiers are in fact the suppressors and enhancers that are present within the natural genetic variation of the species and can affect penetrance, dominance

modification, expressivity and pleiotropy. Epistasis, which occurs when an allele of one gene masks the phenotype of another gene, is one of the genetic explanations for observing the effect of genetic modifiers (Nadeau, 2001). Examples of modifying mutations in Arabidopsis can be found in the work with the *abi1* mutant by Steber et al. (1998) and Beaudoin et al. (2000).

In Arabidopsis most mutational screens are conducted in the standard laboratory strains L*er*, Colombia (Col) and Wassilewskija (Ws). The use of this small number of accessions has limitations since the phenotype that can be identified depends on the wild-type background. For example mutant phenotypes for which the wild-type carries a null or silenced allele might not be detected (Alonso-Blanco and Koornneef, 2000). To overcome this limitation one can study for a given trait genetic variation present among naturally occurring populations. Many traits have already been investigated using natural variation of Arabidopsis (Alonso-Blanco and Koornneef, 2000) and the list is still growing. When exploiting natural variation for seed longevity of orthodox seeds one does not expect to find accessions showing low seed vigor since nature would have selected against such seeds. Therefore an artificial selection system like a CDT or the use of a sensitized background selection seems necessary. Tesnier et al. (2002), showed using a CDT, specially developed for Arabidopsis, that natural variation for seed survival existed since four accessions responded differently to the treatment. Using this test Bentsink et al. (2000) identified, in a (RIL) population between the accessions L*er* and Cvi, four genomic regions affecting seed survival.

The scope of this thesis

Seed quality consisting of seed longevity, vigor and viability, is a complex trait for which seed scientists have been able to identify several factors that are influencing this trait. Seeds are usually stored in conditions that allow as little as possible loss of both vigor and viability. However, differences are observed both between seed lots from the same genotype and between different genotypes. Commercial use of seeds as starting material for crop production requires high performance seeds and thus techniques like priming have been developed to enhance uniform seedling establishment. Little information is known about the genetic factors that regulate seed quality, while this could provide valuable information not only about the relative importance of the various processes involved but it also may assist breeders in improving seed vigor and longevity by breeding. This thesis provides evidence about the complex genetic nature of seed vigor and longevity. The identification of genetic loci controlling this trait in Arabidopsis might serve as a starting point to isolate the respective

genes. This will further increase our knowledge of the genes and processes controlling seed quality.

In Chapter 2 seed longevity of mutants using long-term storage, CDT and germination on H_2O_2 was assessed. A special emphasis was put on mutants disturbed in genes involved in oxidative stress scavenging. Since redundancy was expected “double” mutants were created and thereafter these were used to evaluate the role of ROS in seed quality.

In Chapter 3 an enhancer of the *abi3-1* mutant phenotype, showing a green seed color as a double mutant, is described. The ABA response and dormancy phenotype of the *grs abi3-1* double mutant was not altered however the storability was altered in both the double mutant and the single *grs* mutant. It is concluded that *GRS* could be a common regulator in chlorophyll breakdown and acquiring seed longevity, which are separate processes.

In Chapter 4 *abi3-5* mutants seeds have been used as a “sensitized” background to isolate novel mutants with enhanced seed longevity in this poorly storable genetic background. Furthermore the large genetic variation present among wild Arabidopsis accessions was exploited by identifying modifiers of the sensitive *lec1-3* and *abi3-5* mutations. Lines with enhanced seed longevity, in their respective sensitive mutant backgrounds, have been selected. In total twelve modifiers have been isolated, eight originated from accession selections and four from mutagenesis experiments.

In Chapter 5 a new RIL population between the accessions L*er* and Shakdara is used to identify QTLs related to seed quality. QTLs are identified for CDT survival and germination on salt, mannitol, H_2O_2, ABA and after heat stress. Furthermore, QTLs for other seed properties like dormancy, speed of germination and natural aging have been identified. The possible consequences of co-locations for the various traits are discussed.

Chapter 6 summarizes the results and mentions the research needed to follow up the physiological and molecular characterization of the genes that have been genetically identified in this study.

Chapter 2

Genetic differences in seed longevity of various Arabidopsis mutants

Emile J.M. Clerkx, Hetty Blankestijn-De Vries, Gerda J. Ruys,
Steven P.C. Groot, Maarten Koornneef

Abstract

Seeds gradually loose their viability during dry storage. The damage that occurs can be sustained by the (bio) chemical composition and physiological status of the seeds and is affected by the storage conditions of the seeds. Although these environmental conditions controlling loss of viability have been investigated frequently, little information is available on the genetics of seed longevity. Using Arabidopsis mutants in defined developmental or biochemical pathways such as those affected in seed coat composition, seed dormancy, plant hormone function and mutants with effects on the control of oxidative stress, we tried to get insight into the genes and mechanisms controlling viability of stored seeds. Mutations like *abscisic acid insensitive3* (*abi3*) as well as *abscisic acid deficient1* (*aba1*) show reduced storability, which maybe partially related to the seed dormancy phenotype of these mutations. Mutants with seed coat alterations, especially *aberrant testa shape* (*ats*), showed reduced germination in all tests indicating the importance of a “functional” seed coat for seed longevity. A specific emphasis was put on mutants affected in dealing with Reactive Oxygen Species (ROS). Since several pathways are involved in protection against ROS and because gene redundancy is a common feature in Arabidopsis, “double” mutants were generated. These “double” mutants were subjected to a controlled deterioration test (CDT). Furthermore, a germination assay on hydrogen peroxide (H_2O_2) was performed after prolonged storage at two relative humidities. CDT and germination on H_2O_2 affected all genotypes although it seems that other effects, like the influence of the genetic background, are more important than having a mutational defect in the ROS scavenging pathway. Explanations for this limited effect of mutations affecting ROS scavenging are discussed.

This chapter has been submitted for publication

Introduction

Seeds of good quality are undamaged and have a high level of germination also after storage and therefore will produce vigorous seedlings without defects under various environmental conditions (Dickson, 1980). Seed quality can be reduced on the parental plant due to adverse environmental conditions, premature germination (Coolbear, 1995) and pathogens (McGee, 2000). Physiological damage can be of different types, e.g. short-term deterioration in the field is different from long-term deterioration during storage, which in turn is different from sustained mechanical damage (McDonald, 1999). All seed organs can suffer physiological damage: the seed coat, which is of maternal origin, the embryo and the endosperm. Damage can be sustained by the chemical and biochemical composition of the seeds and by the way these compounds make up biological structures in seeds. Especially the integrity of DNA, proteins and membranes is important for maintaining the viability of seeds.

Tolerance to desiccation is a survival mechanism adopted by orthodox seeds (Wilson Jr., 1995) and develops during the early phases of seed maturation after morphogenesis is completed (Bewley and Black, 1994). Upon dry storage, seeds gradually loose their viability. During the last period of seed maturation, well beyond the moment desiccation tolerance has been acquired, seeds still gain in longevity (Hay et al., 1997; Jalink et al., 1998). Seeds that have initiated germination processes, e.g. during seed priming or on the motherplant, also exhibit reduced storability (Groot et al., in press). Mutants that have a defective seed development such as *leafy cotyledon1* and *2* (*lec1*, *lec2*), *fusca3* (*fus3*) and extreme alleles of *abi3* exhibit an extremely short storability, since they loose their viability within a few weeks of dry storage (Clerkx et al., Chapter 4). In general the rate of viability loss depends very much on the storage conditions; especially seed moisture content, which is depending on the relative humidity (RH), and temperature of the environment are important factors (Ellis et al., 1990; Bewley and Black, 1994). Hay et al. (2003) predicted that in dry and cold conditions, 5% moisture content and –20°C, Arabidopsis seeds should be storable for approximately 2000 years.

Although the environmental conditions that control loss of viability and vigor have been investigated frequently, little information is available on the exact mechanisms that control this loss of viability. Most data in literature concerning this process comes from studies where loss of viability was correlated with biochemical changes and also by assuming that certain cellular stress generating processes are important.

The use of mutants related to defined developmental or biochemical pathways may be helpful in this because it can lead to conclusions about which processes are more and which are less important for seed longevity. Here we have used a number of Arabidopsis

mutants to study seed quality loss upon dry storage. Mutants were chosen based on their effect on seed dormancy, plant hormone biosynthesis or signal transduction, on their effect on accumulating or avoiding oxidative stress, or because they have a defect in their seed coat. Plant hormones may influence germination/dormancy as shown for abscisic acid, gibberellin, and ethylene. Furthermore the response to environmental stress is also influenced by plant hormones, as shown for the response to oxidative stress by jasmonic acid and ethylene (Overmyer et al., 2003). Seed coat mutants were included in this analysis because some reduction in seed vigor has been described for such mutants by Debeaujon et al. (2000).

A specific emphasis was put on mutations affecting oxidative stress because it is often reported that a common cause of the damage might be the production of free radicals (Hendry et al., 1993; McDonald 1999) attacking cellular components. Free radicals can be generated by carbon, sulfur, phosphorus, nitrogen, iron and manganese metabolism and may owe their biological origin ultimately from the radical promoted transfer of electrons to and from oxygen (Hendry, 1993). ROS are a.o. the superoxide and hydroxyl molecules and, although not a true radical, H_2O_2, since it can provide, through the Fenton reaction, hydroxyl molecules. ROS are generated in both stressed and unstressed cells, and in various cellular compartments. They are generated endogenously during certain developmental transitions such as seed maturation and as a result of normal, unstressed photosynthetic and respiratory metabolism (Grene, 2002). In seeds the most likely origins are the peroxysomes, the mitochondria, autoxidation in the cytosol and through chlorophyll, which is normally degraded during maturation. ROS could directly, or via subsequent lipid peroxidation, lead to mitochondrial dysfunction, enzyme inactivation, membrane perturbation and genetic damage (Coolbear, 1995). In addition to the amount of ROS generated, the final damage is also determined by the efficiency of the cells, in which these reactive molecules are formed, to scavenge and dispose of them.

The antioxidant defense system in plants consists of both enzymatic and non-enzymatic antioxidants. It should be realized that the enzymatic antioxidant system can be only active under conditions of sufficient water. In the quiescent period when seeds are dehydrated, only molecular antioxidants (e.g. glutathione, ascorbate, polyols, carbohydrates, peroxyredoxin, tocopherol and phenolics) can alleviate oxidative stress (Hoekstra et al., 2001).

Superoxide dismutase (SOD) (Kliebenstein et al., 1998; Grene, 2002) catalyzes the conversion of superoxide radical to H_2O_2. Hydrogen peroxide can be disposed of by catalase and ascorbate peroxidase. Catalases convert H_2O_2 to water and oxygen, ascorbate peroxidase forms water and dehydroascorbate from ascorbic acid (vitamin C) and H_2O_2 (Willekens et al., 1995; Noctor and Foyer, 1998; Blokhina et al., 2003). Futhermore H_2O_2

removal via ascorbate peroxidase requires glutathione since ascorbate is reduced by dehydroascorbate reductase using glutathione (GSH) as a reducing substrate. This process is known as the ascorbate-glutathione cycle (Noctor and Foyer, 1998). Other antioxidants such as vitamin E (α-tocopherol) act against phospholipid radicals (Grene, 2002). Phenolic compounds, among which flavonoids, are abundant in plant tissues (reviewed by Grace and Logan, 2000) and their antioxidant properties arise from their high reactivity as hydrogen or electron donor and from the ability of the polyphenol-derived radicals to stabilize and delocalize the unpaired electron. Another function lies in their ability to chelate transition metal ions and thus terminate the Fenton reaction (Rice-Evans et al., 1997). The seed coat itself, which in its mature state contains tannins, that are oxidized flavonoid polymers, may play a role in depriving the embryo from oxygen during storage and thus preventing ROS formation (Corbineau and Come, 1993).

The study of ROS mediated damage and repair in seeds is complex, especially when they are in a dehydrated state. Adding water to dry seeds could start germination processes, which subsequently activate the antioxidant defense mechanism. In a large number of publications correlative evidence is provided that both the amount of oxidants and the antioxidant activity correlate with seed quality. Senaratna et al. (1988) showed that aged soybean axes had a low antioxidant potential when imbibed, indicating that the aging process was associated with exposure to oxidative stress. Puntarulo et al. (1991) showed an increase in both oxidants and antioxidants during germination of soybean and similar observations were made during germination in *Pinus pinea* L. (Tommasi et al. 2001), wheat (Cakmak et al. 1993) and *Zea mays* L. (Leprince et al., 1994). Recalcitrant seeds, which are desiccation intolerant, contain higher amounts of ascorbic acid compared to orthodox seeds. This also might be an indication for the importance of scavenging free radicals to prevent seed aging. Apparently moist recalcitrant seeds need a constant high level of protection while dry orthodox seeds have a low metabolic activity and hence a relatively low production of hydrogen peroxide (Tommasi et al., 1999). Bailly et al. (1996) showed that the faster deterioration of sunflower seeds during accelerated aging, compared to non-aged controls, was related to a decrease in enzymes involved in ROS scavenging.

Plant hormones play a role in programmed cell death (PCD) mediated by oxidative stress and the systems used to elucidate the role of hormones in this process are the hypersensitive response (HR) to pathogens and the exposure to ozone (O_3) (Overmeyer et al., 2003). Plant hormones like ethylene and salicylic acid enhance the accumulation of ROS in PCD and in ROS dependent lesion propagation, whereas jasmonic acid is involved in ROS containment, as was concluded from the observation that *ethylene resistant1* (*etr1*) mutants are more tolerant while *jasmonic acid resistant1* (*jar1*) mutants are more sensitive to ozone (Overmeyer et al., 2003). However, it has also been reported that ethylene, salicylic acid and

abscisic acid are involved in protection of plants against heat induced oxidative damage since mutants like *etr1*, *abi1* and the salycilic acid deficient (nahG) mutant are more sensitive to this stress (Larkindale and Knight, 2002). Besides in heat stress, abscisic acid plays a central role in stress signaling in both biotic and abiotic stresses (reviewed by Xiong et al., 2002).

Here we report a study of mutant seeds that have been stored under "normal" laboratory conditions (ambient temperature and RH) for four years and in addition a subgroup has been tested with a CDT, developed for Arabidopsis by Tesnier et al. (2002) that mimics natural aging. The effect of ROS was tested using the *frostbite1* (*fro1*) mutant, recently described as having high constitutive levels of ROS (Lee et al., 2002) and by germination on H_2O_2. Superoxide radicals are believed not to be able to pass biological membranes (Grene, 2002) and are very short lived. Therefore a germination assay on H_2O_2 was used; H_2O_2 is normally formed as a result of SOD action and is capable of diffusing across membranes (Grene, 2002).

A specific problem that we encountered in this genetics approach is that the various mutants are in different genetic backgrounds. Different accessions show different seed quality properties as was shown by their response to a CDT indicating the existence of genetic variation for the response to this test (Bentsink et al., 2000; Tesnier et al., 2002; Clerkx et al. Chapter 5).

Results

Germination of "old" seed lots

A germination test was performed with four year old seed lots of a number of monogenic mutants. The seeds that had been stored under ambient conditions showed differences in survival (Fig. 2.1). All seeds had been produced in the same greenhouse under the same conditions and were harvested at the same time except the three Landsberg *erecta* (L*er*) seed lots, which had been harvested a few weeks earlier. After a four year storage period some of the L*er* seeds failed to germinate. The most severely affected mutants were those with the different *abi3* alleles in L*er* background. Significantly reduced germination was also observed for the *ats* mutant in L*er* background, which confirmed earlier observations in our laboratory. The decreased germination of other mutant seeds like *gibberellin insensitive* (*gai*), *reduced dormancy2* (*rdo*) and *gibberellin deficient1-3* (*ga1-3*) was not significantly different from the wild-type L*er*. A decreased germination, for mutants in Colombia (Col) background was observed for C3-7-1 (reduced dormancy), *etr1* and *aba1-5*, although the only significant reduction was observed for the latter. These results indicated an important

role for ABA in seed storability, and possibly a role for seed dormancy, which is also strongly reduced in ABA related mutants. No effect was observed for the two mutants affected in ROS scavenging *vitamin-C deficient1-1* (*vtc1-1*) and *cadmium sensitive2-1* (*cad2-1*), the latter being glutathione deficient.

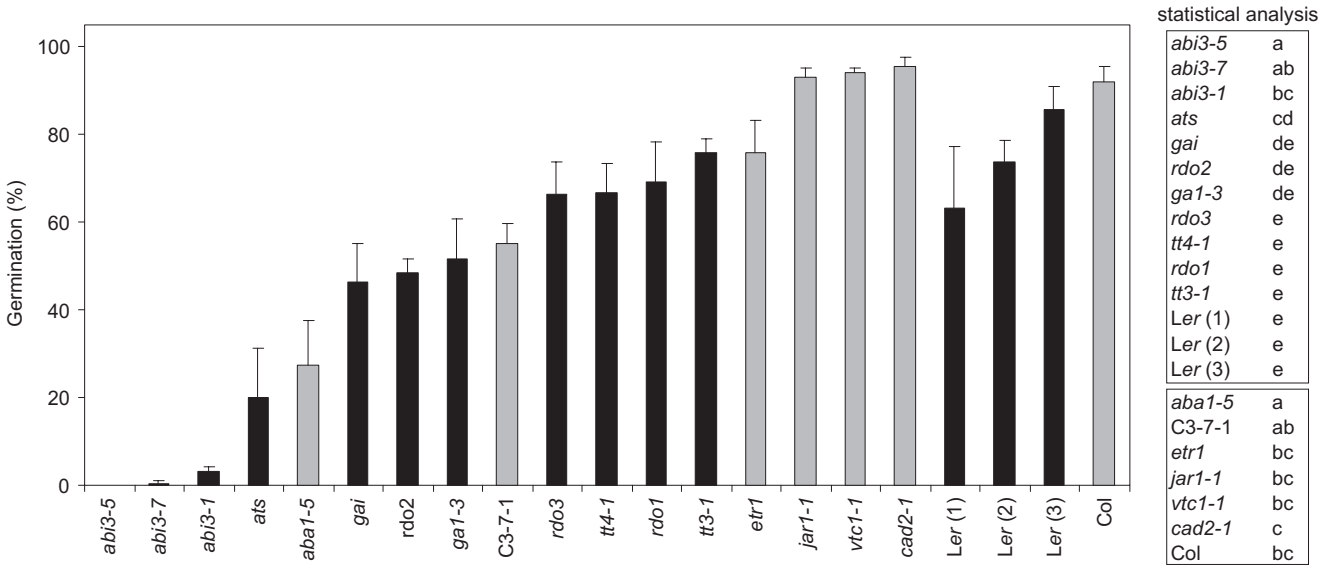

Figure 2.1: Germination percentages (+ SE) and statistical analysis of hormone, dormancy and oxidative stress related mutants and their respective wild-types after four years of storage. L*er* (1) till (3) represent three different seed batches. Mutants in L*er* background and L*er* wild-type are depicted as black bars and mutants in Col background and Col wild-type are depicted in gray bars. Each mutant indicated with a common letter could not be separated statistically ($P < 0.05$). Statistical analysis was performed only among their respective genetic background, top square L*er* comparisons and bottom square Col.

The effect of ROS accumulation

To investigate if ROS induced damage may be an important component of reduced viability upon Arabidopsis seed storage a CDT was performed with the *fro1* mutant of which mutant leaves accumulate ROS constitutively. To test what the effect of this mutation was on CDT survival the mutant and controls (C24 and RD29A::LUC, Table 2.3 in Materials and Methods) were subjected to 1 day of controlled deterioration treatment (Fig. 2.2). It appeared that this mutant was, significantly ($P< 0.05$), more sensitive to the treatment compared to the corresponding controls. The total number of germinated seeds decreased and the number of abnormal seedlings as percentage of total germination increased.

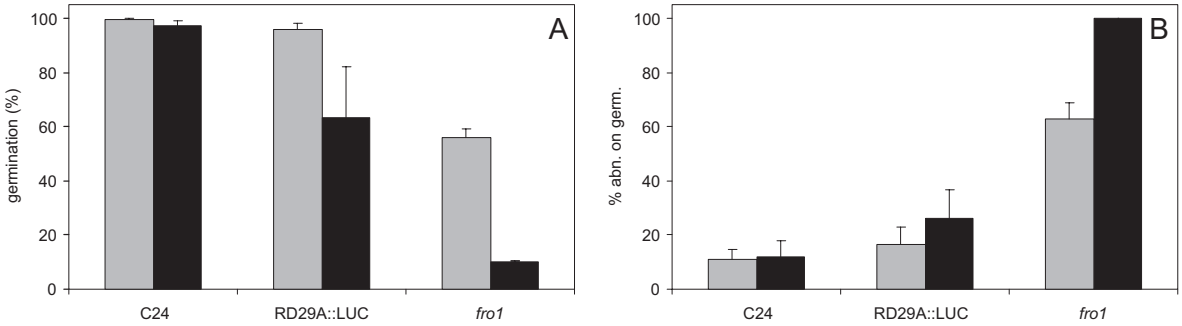

Figure 2.2: Germination percentages (A) and percentage of abnormal seedlings of total germination (B) of the controls C24 and RD29A::LUC and *fro-1* after 0 (gray bars) and 1 (black bars) day CD treatment (+ SE)

The effect of CDT on mutants and "double" mutants with antioxidant related phenotypes.

To analyze the effect of various mutations on seed quality in detail, a CDT was performed with seeds harvested from mature dry siliques that had been stored at ambient conditions for 9 months. Since residual dormancy was not expected, no dormancy breaking treatment was applied prior to germination. The results however showed that in seeds of Wassilewskija (Ws) and of the double mutant *cat1 cat3*, in Ws background, dormancy was still present since the germination increased with the duration of the CD treatment (CDT1, Fig. 2.3A). The same effect was observed in Col and the *vtc1-1* and *cad2-1* mutants in Col background. To test whether this reduced germination was due to residual dormancy, or induced by the treatment, several mutants and wild-types were tested for the effect of a cold treatment. This experiment showed that some dormancy was still present in a number of these lines, but it also showed that one week of cold treatment should be sufficient to break the residual dormancy (data not shown). A second CDT (CDT2, Fig. 2.3B) was performed and a dormancy breaking treatment was applied by imbibing the seed for 7 days at 4°C prior to the germination assay. Again the Ws and the *cat1 cat3* mutant showed reduced germination, although less than without cold treatment. The cold treatment was enough to break dormancy of Col and the mutants in Col background, *vtc1-1* and *cad2-1*. Since Ws and *cat1 cat3* mutants germinated fully after cold treatment in a germination assay prior to CDT2 (data not shown) it was concluded that the observed dormancy was induced by the treatment. Although in both CDT1 and CDT2 induced dormancy was observed, the effect of the CDT was obvious for all genotypes. The usefulness of the test became clear when the germination of *vtc1-1*, *cad2-1*, Col and L*er* after CDT was compared to germination after four years of normal storage (Fig. 2.1). The CDT reduced the viability of these genotypes fast while a four year storage only slightly decreased viability. Deterioration was estimated as a

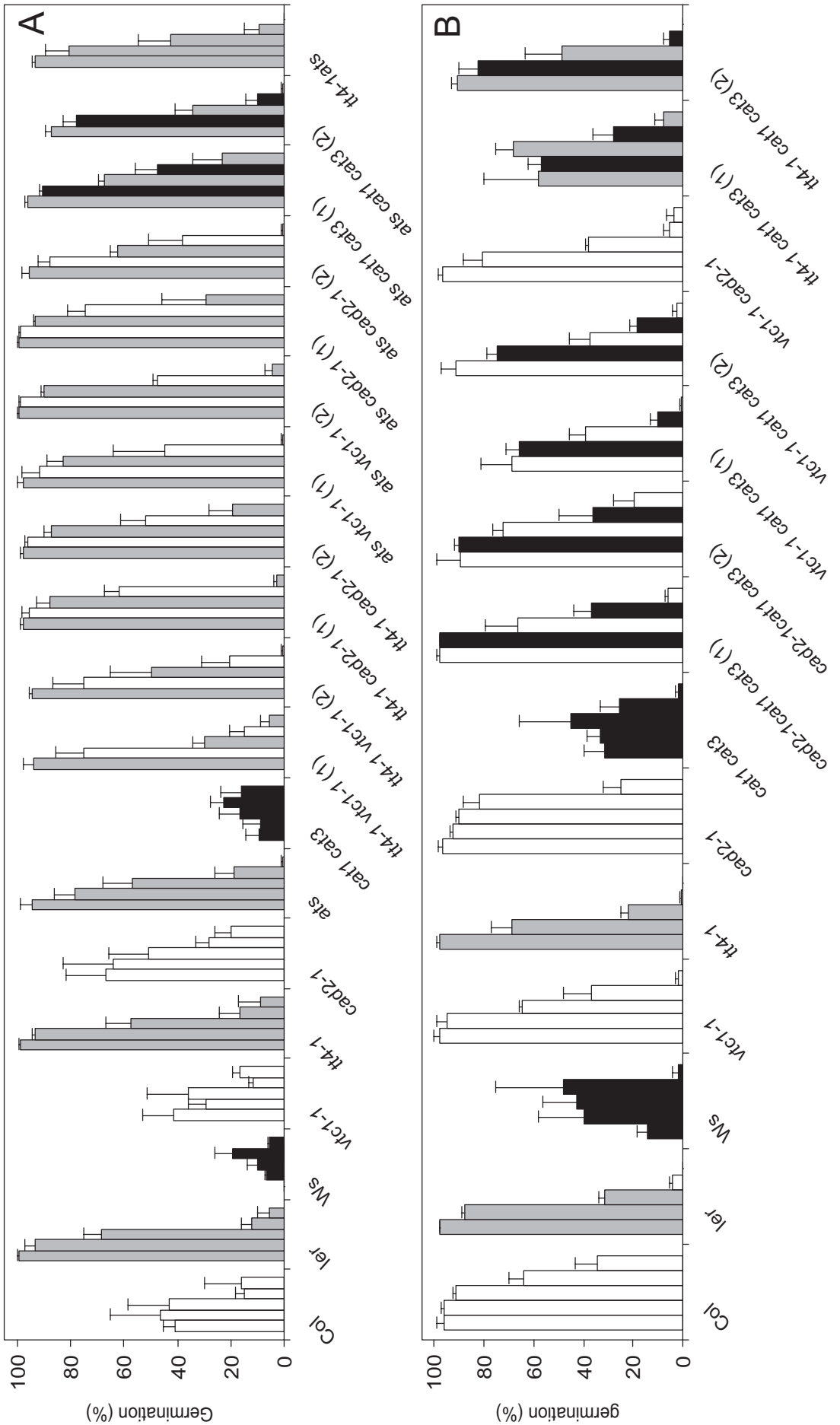

Figure 2.3: Germination percentages after 0, 1, 3, 5 and 7 days of controlled deterioration treatment + SE of CDT1 (A) and CDT2 (B). Colors indicate the genetic background of the various wild-types, mutants and "double" mutants, Col: white, L*er*: gray and Ws: black, mixed graphs indicate mixed backgrounds: gray/white is L*er*/Col, gray/black: L*er*/Ws and white/black: Col/Ws.

single parameter: D_{50}, the number of days of deterioration treatment required to reach 50% germination and this parameter was also used in statistical analysis. Some “double” mutants showed a significant reduction in D_{50} values compared to their respective wild-type or single mutant controls (Table 2.1, CDT 1 and CDT2). However none of the “double” mutants showed a significant reduction to all its controls also because some comparisons could not be made due to the induced dormancy.

Table 2.1: Comparison of CDT, each mutant was compared to the respective controls statistical analysis was performed using the D_{50} values

CDT 1

	D_{50}	std err T_{50}	Col	L*er*	Ws	*vtc1-1*	*tt4-1*	*cad2-1*	*ats*	*cat1 cat3*
vtc1-1	n.d.		= (d)#							
cad2-1	n.d.		= (d)#							
tt4-1	3.58	0.23		=						
ats	3.05	0.43		=						
cat1 cat3	n.d.				+ (d)					
tt4-1 vtc1-1 (1)	2.55	0.34	- (d)#	-		- (d)	-			
tt4-1 vtc1-1 (2)	2.92	0.65	- (d)#	-		- (d)	-			
tt4-1 cad2-1 (1)	4.88	0.18	+ (d)#	+			+	+ (d)#		
tt4-1 cad2-1 (2)	5.21	0.37	+ (d)#	+			+	+ (d)#		
ats vtc1-1 (1)	4.19	0.47	+ (d)#	+		+ (d)			+	
ats vtc1-1 (2)	4.72	0.13	+ (d)#	+		+ (d)			+	
ats cad2-1 (1)	6.27	0.56	+ (d)#	+				+ (d)#	+*	
ats cad2-1 (2)	3.72	0.37	+ (d)#	=				+ (d)#	+	
ats cat1 cat3 (1)	4.88	0.62		+	+ (d)				+	+ (d)#
ats cat1 cat3 (2)	2.33	0.32		-	- (d)				-	- (d)#
tt4-1ats	2.61	0.49		-			-		-	

CDT 2

			Col	L*er*	Ws	*vtc1-1*	*tt4-1*	*cad2-1*		*cat1 cat3*
vtc1-1	3.95	0.28	-							
cad2-1	6.21	0.56	=							
tt4-1	1.92	0.20		-						
cat1 cat3	n.d.				- (d)#					
cad2-1cat1 cat3 (1)	4.15	0.35	-		= (d)#			-		= (d)#
cad2-1cat1 cat3 (2)	4.53	0.60	-		= (d)#			-		= (d)#
vtc1-1 cat1 cat3 (1)	2.15	0.09	-*		- (d)#	-*				- (d)#
vtc1-1 cat1 cat3 (2)	2.70	0.20	-*		- (d)#	-				- (d)#
vtc1-1 cad2-1	2.77	0.38	-*			-		-*		
tt4-1 cat1 cat3 (1)	3.94	0.41		+	= (d)#		+*			= (d)#
tt4-1 cat1 cat3 (2)	2.60	0.42		=	- (d)#		+			- (d)#

-: indicates a worse germination of the mutant in the column compared to the respective genotype at the top. =: no difference in germination was observed; +: a better germination was observed; n.d.: not determined due to dormancy. If dormancy was present germination differences were estimated based on day 5 and 7 of treatment. Statistical analysis was performed using the D_{50} values: *indicates a statistical significant difference. #: indicates no statistical analysis was performed using this data; (d): indicates dormancy was present in this line so statistical comparison is not possible. A correction to the P value for multiple tests was made: CDT1 $P < 0.008$ and CDT2 $P < 0.012$ was considered different D_{50} values for L*er* are CDT1: 3.62 ± SE of 0.15 and CDT2 2.49 ± SE of 0.07 and Col in CDT 2 6.08 ± SE of 0.41

The effect of CDT on the *ats* mutant

After four years of storage the *ats* mutant seeds showed a significant reduction in germination compared to the L*er* wild-type, although in the CDT the reduction was not significant (Table 2.1, CDT1). To test whether the reduction in germination, in *ats* seeds, was embryonic or due to the maternally inherited seed coat defect, reciprocal F_1 seeds between L*er* and *ats* were made. The F_1 and control seeds were subjected to a CD treatment. Since the seed coat is of maternal origin, F_1 seeds had either the wild-type seed coat (F_1 L*er* x *ats*) or the mutant seed coat (F_1 *ats* x L*er*) while the embryo is heterozygous. The deterioration pattern of the F_1 L*er* x *ats* seeds resembled more that of the L*er* seeds, whereas that of the F_1 *ats* x L*er* resembled that of *ats* seeds (Fig. 2.4). Therefore it seemed likely that the aberrant seed coat increased the sensitivity to CDT.

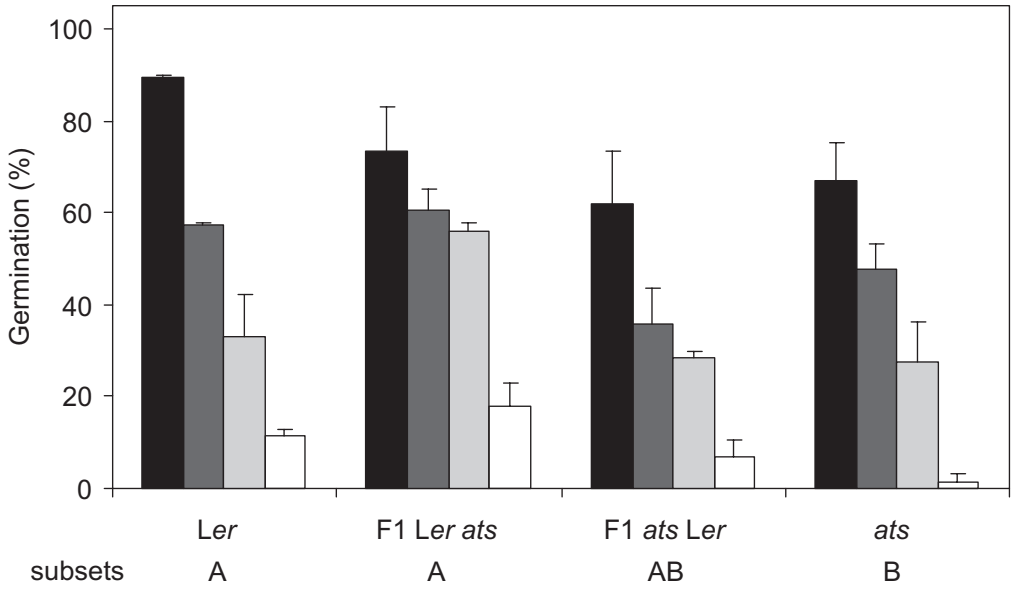

Figure 2.4: Germination percentages + SE of L*er*, F_1 L*er ats*, F_1 *ats* L*er* and *ats* after 0 (black bars), 1 (dark gray bars), 2 (light gray bars) and 4 (white bars) days of controlled deterioration treatment. Common letters indicate that values could not be separated statistically ($p < 0.05$)

The effect on germination of hydrogenperoxide (H_2O_2)

It could be expected that mutants with a defective antioxidant function should be more sensitive to the inhibiting effect of a ROS generating compound such as H_2O_2. Seeds that had been stored for 11 months at ambient conditions and thereafter for one year either at 32% or 60%RH were imbibed and left to germinate at two concentration of H_2O_2 (Fig. 2.5 A and B). Most lines showed 100% or almost 100% germination upon imbibition on water. There was a slight reduction in the *cat1 cat3* double mutant seeds and in seeds of lines that

had the *ats* mutant phenotype. Imbibition and subsequent germination on H_2O_2 strongly reduced germination of all seeds, especially when 0.5% H_2O_2 was applied. Storage at 60% RH renders seeds more sensitive to H_2O_2. The *ats* mutant was very sensitive to H_2O_2, even the lowest concentration 0.3% H_2O_2 (gray bars in 2.5A and B) prevented germination completely. This effect was also observed in all "double" mutants with *ats*, where the effect of the aberrant testa is so strong that the effect a second mutation cannot be seen.

Statistical analysis using the probit transformed germination data after 0.3% H_2O_2 treatment are compiled in Table 2.2, with analyses after 0.5% H_2O_2 giving similar results. Comparing the *vtc1-1*, *cad2-1* and *cat1 cat3* mutants and the respective "double" mutants showed that none of them is significantly different from its controls. The *tt4-1* mutant seeds germinated significantly less than L*er* seeds after storage at 32% RH and germination on 0.3% H_2O_2. A decrease in germination was also observed after 60%RH storage, although not significant. The overall tendency for "double" mutants, which combine *tt4-1* with the ROS scavenging deficiency mutants, suggested an additive effect, although only one of the *tt4-1 cat1 cat3* lines was significantly different from its respective controls after 32%RH storage.

Table 2.2: Comparison of germination percentages and statistical analysis of all mutants and double mutants after storage at 32% and 60%RH and subsequent germination at 0.3%H_2O_2.

	Col		L*er*		Ws		*vtc1-1*		*tt4-1*		*cad2-1*		*ats*		*cat1 cat3*	
storage condition (%RH)	32	60	32	60	32	60	32	60	32	60	32	60	32	60	32	60
vtc1-1	=	+														
cad2-1	-	+														
tt4-1			-*	-												
ats			-*	-*												
cat1 cat3					=	+										
cad2-1cat1 cat3 (1)	-	+			-	-					=	=			-	-
cad2-1cat1 cat3 (2)	-	=			-	-					=	-			-	-
vtc1-1 cat1 cat3 (1)	=	+			-	+	=	+							-	=
vtc1-1 cat1 cat3 (2)	-	+			-	=	-	+							-	-
vtc1-1 cad2-1	-	+					=	+			=	+				
tt4-1 vtc1-1 (1)	-	-	-	-			-	-	-	-						
tt4-1 vtc1-1 (2)	-*	-	-	-			-*	-*	-	-*						
tt4-1 cad2-1 (1)	-	-	-	-					-	-	-	-				
tt4-1 cad2-1 (2)	-	-	-	-					-	-	-	-				
tt4-1 cat1 cat3 (1)			-*	-	-*	-*			-	-					-*	-*
tt4-1 cat1 cat3 (2)			-*	-*	-*	-*			-*	-					-*	-*
ats vtc1-1 (1)	-*	-*	-*	-*			-*	-*					u	u		
ats vtc1-1 (2)	-*	-*	-*	-*			-*	-*					u	u		
ats cad2-1 (1)	-*	-*	-*	-*							-*	-*	u	u		
ats cad2-1 (2)	-*	-*	-*	-*							-*	-*	u	u		
ats cat1 cat3 (1)			-*	-*	-*	-*							u	u	-*	-*
ats cat1 cat3 (2)			-*	-*	-*	-*							u	u	-*	-*
tt4-1ats			-*	-*					-*	-*			u	u		

u: indicates that no differences can be detected because these seeds did not germinate at 0.3% H_2O_2. At the top of the table all genotypes the comparison was made to; -: indicates a lower germination; +: indicates a better germination;=: indicates that no difference in germination could be observed and *: indicates a significant difference. Statistical analysis was performed with probit transformed data, the $P < 0.005$ (corrected for multiple tests) was considered significant.

Figure 2.5: Germination percentages of controls, single mutants and "double" mutants after 11 months storage at ambient conditions and storage for 1 year at 32%RH (A) and 60%RH (B) and subsequent germination on 0% H_2O_2 (black bars), 0.3% H_2O_2 (gray bars) and 0.5% H_2O_2 +SE

Discussion

Differences in seed quality are often observed when seed batches are compared. Most emphasis in research on seed quality has been focused on the comparison of seed batches of similar genotypes in different physiological environments among which temperature and RH during storage are the most important (Bewley and Black, 1994; Coolbear, 1995). In the present study we analyzed different genotypes but maintained similar environmental conditions by growing seeds in the same experiments and by storing the genotypes under the same conditions. Deterioration of seed was achieved by prolonged storage in ambient conditions, by storage at higher RH and by the application of a CDT, developed for Arabidopsis by Tesnier et al. (2002), since it allows a quick deterioration of genotypes that under natural storage conditions remain viable for at least four years.

The comparison of germination percentages after four of year storage of various mutants selected because of their effect on seed germination, their effect on hormone biosynthesis or hormone action and because of their role in the protection against oxidative stress, indicated that considerable differences exist in comparison to their corresponding wild-types. For extreme *abi3* alleles, like *abi3-5*, (Ooms et al. 1993, Bies-Ethève et al. 1999) poor storability has been described before. However, it has not been reported for the leaky *abi3* alleles, like *abi3-1* and *abi3-7* (Bies-Ethève et al., 1999), which were more sensitive than wild-type to a CDT (Tesnier et al., 2002; Clerkx et al., 2003). Other mutants that showed reduced storability are *ats*, *aba1-5* and possibly *rdo2* and C3-7-1, which have in common with the *abi3* alleles a reduction in seed dormancy. During dormancy metabolism is low, hence a low production of detrimental products might improve longevity. Bentsink et al. (2000) and Clerkx et al. (Chapter 5) showed, by QTL mapping, that the more dormant Arabidopsis accession Cape Verde Islands (Cvi) and Shakdara had a higher survival after a CDT.

The *ga1-3* and *etr1* mutants show a reduced germination because dormancy breaking is relatively ineffective (Koornneef and van der Veen, 1980; Koornneef et al., 2000). Especially for the *ga1-3* the antagonistic interaction with the *aba1* mutant is well documented (Debeaujon and Koornneef 2000). This antagonistic effect was not observed for seed storability.

Another indication of a relationship between dormancy and seed longevity is the induction of secondary dormancy, which in nature is a response to unfavorable germination conditions (Bewley and Black, 1994). In CDT1 in Col, *vtc1-1*, *cad2-1*, Ws and *cat1 cat3* and in CDT2 in Ws and *cat1 cat3* secondary dormancy is induced. The dormancy might be induced in two ways either through the high temperature at which the seeds are exposed during the treatment (40°C) or by re-drying the seeds. The first is known as thermodormancy,

induction of dormancy at a temperature above the optimal germination temperature (Bewley and Black, 1994). Thermodormancy is not the likely cause of induction since control seeds (0 days treatment) are only equilibrated at 85% RH at 15°C and not exposed to 40°C, while these seeds do show induction of secondary dormancy. The second, more likely, cause is the drying of the seeds after treating them with high temperature at relatively high humidity (85% RH) when the seeds are dried back to 32% RH, resulting in a seed moisture content (MC) of approximately 6% (Tesnier et al., 2002). All seeds are then stored at 4°C, and 6% MC, until sowing of all seeds at the same day. The induction of secondary dormancy due to drying is reported for switchgrass seeds that had been stratified. Immediately after stratification the germinability was high, but when followed by slow drying the germination of the seeds decreased again (Shen et al., 2001).

During the hypersensitive response (HR) after pathogen infection and ozone induced cell death, involving ROS, the mutant *etr1*, which is defective in the ethylene receptor, should not enhance the production of ROS, whereas the *jar1* mutant, which is not able to contain the spreading of cell death enhances the effects of these treatments (Overmyer et al., 2003). If these hormones would play a role in ROS accumulation during long-term seed storage one would expect that *etr1* shows an enhanced seed longevity whereas *jar1* would show the opposite effect. We were not able to confirm this hypothesis since neither *etr1* nor *jar1* differed significantly from their wild-type background Col (Fig. 2.1) while the germination percentages might even suggest the opposite to the predicted effects.

The effect of the monogenic mutants in which antioxidant levels are reduced is limited. It was expected that such mutants would be affected by long-term storage and CDT conditions since several authors have suggested that ROS damage could be a major cause of seed damage during storage (Hendry, 1993; McDonald, 1999) especially in oil containing seeds where lipid peroxidation can generate ROS. However, it has also been suggested that the oil itself may undergo lipid peroxidation and in this way protect membranes from damage (Gidrol et al., 1989). Damage related to ROS, and the way this is affected by environmental and genetic factors, can be seen as the sum of the amount of ROS generated and the degree by which it is curtailed. An important aspect of the latter is the effectiveness of the enzymatic and non-enzymatic antioxidant defense systems but also the possibility to slow down metabolism in general. CDT is known to generate free radicals (Bailly et al., 1996; Khan et al., 1996) but also has been shown to affect the level of antioxidants (De Vos et al., 1994; De Paula et al., 1996; Bailly et al., 1997). Oxidative stress can reduce seed quality, which is suggested by the decline in germination of the *fro1* mutant, known to accumulate ROS constitutively (Lee et al. 2002). A problem with the interpretation of the single mutant phenotypes, especially when related to the antioxidant effects is the functional redundancy of the different antioxidant systems. This relates to the multitude of both enzymatic and non-

enzymatic antioxidant systems of which the first is only functional when sufficient water is available (Hoekstra et al., 2001). It also relates to the fact that most of the mutants used are 'leaky', which means that a residual amount of antioxidants is present. The *cat1 cat3* double mutant still has a functional *CATALASE2* gene. The ascorbic acid deficient mutant (*vtc1-1*) still has 30% of the wild-type levels left (Conklin et al., 1996) and also the glutathion deficient mutant (*cad2-1*) still contains 15-30% of glutathion compared to wild-type (Howden et al., 1995).

For a better understanding of the importance of the antioxidant systems we generated "double" mutants, assuming that a synergistic effect might be observed in some "double" mutants when the total level of important antioxidants would drop below a certain threshold. Although some of the "double" mutants might show a reduced survival in both the CDT and the hydrogenperoxide germination, they are not extremely sensitive. Three possible explanations for this limited effect can be given. Firstly ROS accumulation and the level of antioxidants are of minor importance in generating damage during seed storage, when no excessive ROS is generated which is probably the case in the *fro1* mutant. Secondly the mutant combinations studied still have antioxidant levels above the critical threshold, especially in seeds where these antioxidants have not been analyzed. Measuring ascorbate and glutathion levels, more particularly their redox state, could give insight in this aspect. Thirdly other protection mechanisms might be equally, or more, important. These may be other antioxidant systems such as α-tocopherol, the level of SOD (reviewed by Grene, 2002) and *AtPer1*, which is a seed specific peroxiredoxin maintained in the dry seed (Haslekas et al., 1998). Important factors might also be structural factors, such as the effectiveness by which membranes and other macromolecules are protected. A role for sugars has been suggested and a low ratio of sucrose to oligosaccharides was found to correlate with long-term storability of seeds (Obendorf, 1997). Other protective mechanisms important in dry seeds are the accumulation of amphiphilic molecules such as late embryogenic abundant (LEA) proteins, fructans and the successful formation of a biological glass protecting macromolecules and structural components (reviewed by Oliver et al., 2001 and Hoekstra et al., 2001). The glassy state of desiccation tolerant tissue is depending on temperature and hydration state. Leprince and Hoekstra (1998) showed that during seed dehydration, metabolism is down regulated due to an increasing viscosity of the cytoplasm, which could prevent ROS production.

The reduction in germination of the testa single and "double" mutants in the H_2O_2 germination is stronger than the effect of the antioxidant mutants. The *ats* mutant is characterized by the absence of two out of five integument layers (Léon-Kloosterziel et al., 1994) and *tt4-1* mutant seeds are characterized by the absence of tannins in their seed coat and both mutations enhance the permeability of the testa (Debeaujon et al., 2000). These

mutants might therefore be much more permeable for H_2O_2 compared to mutants with normal seed coats since all “double” mutants with an *ats* mutant testa do not germinate on the concentrations H_2O_2 employed while all *tt4-1* seeds seem to show a reduction in germination. The decrease of viability of *ats* mutant seeds is also observed after four year storage (Fig. 2.1), indicating the importance of a “functional” seed coat for seed longevity, even during natural aging. However, the fact that an effect of the *ats* mutant in the CDT is relatively small, whereas natural aging has a larger effect might suggest that a CDT does not mimic natural aging completely.

Another outcome of our analyses is that the genetic background, that is the accession, in which a mutation is present, plays an important role. This is indicated by the observation that when the same two mutations are combined in various combinations of mixed genetic backgrounds, they show a different reaction to the same treatment. These differences between two “double” mutant lines, carrying the same mutations are observed both in the CDT and in the germination on H_2O_2. Allelic differences between accessions could be observed when quantitative traits loci (QTL) for CD test survival were mapped in L*er*/Cvi (Bentsink et al., 2000) and in L*er*/Sha (Clerkx et al., Chapter 5) recombinant inbred line populations. Allelic differences in response to oxidative stress, induced by paraquat, were also observed by Abarca et al. (2001) who found that the Cvi accession was more tolerant to this treatment compared to L*er* and Col. It was suggested that this difference in tolerance could be explained at least partially by a different allele of a Cu/Zn SOD. These observations make studying the effects of defects in antioxidant scavengers in the same genetic background necessary, especially when the effect of these mutants is relatively small as found in the present study.

Materials and methods

Plant Material and selection of “double” mutants

Here a set of mutants was used the defects, their respective wild types and references can be found in Table 2.3. The F_1 seeds of L*er* x *ats* and *ats* x L*er* were generated by crossing the respective mutant and wild-type line; the control L*er* and *ats* seeds, used in the same experiments, were derived by hand pollination as well. The *frostbite 1* (*fro1*) mutant was isolated in the RD29A::LUC background this reporter line emits bioluminescence in response to low-temperature, ABA or NaCl treatment. The *fro1* mutant was isolated from an EMS treated RD29A::LUC M2 population because it showed a lower level of luminescence under low temperature treatment. Characterization of the *fro1* mutant showed a constitutive accumulation of ROS (Lee et al., 2002).

“Double” mutants, with oxidative stress scavenger defects, were obtained by crossing the individual mutants among each other. “Double” mutants were selected from at least 200 individual F2 plants derived from these crosses. Selection of the “double” mutants was either done based on their phenotype (*tt4-1* and *ats*) or with the use of mutant allele specific PCR-markers. Table 2.4 contains all marker information. A CAPS marker identifying catalase 1 was developed based on the catalase 1 sequence (catalase 1:genbank accession no. U43340). The catalase 3 primers are described by Frugoli et al. (1996). The catalase double mutant has a large deletion (McClung pers. comm.), which enables identification of this mutant by the absence of amplification of both PCR products for *CAT1* and *CAT3*, while in the wild-type both products are present. For the glutathione deficient mutant *cad2-1*, a CAPS marker was developed on the basis of the published mutation (Cobbett et al., 1998), which amplified part of the gene and for which a subsequent digestion, with BSL1 at 55°C, distinguished the mutant from the wild-type allele. No CAPS marker could be made to differentiate between the *vtc1-1* and *VTC1* alleles and therefore the point mutation (Conklin et al., 1999), was used to develop a dCAPS marker (Neff et al., 1998), for which the enzyme BamH1 will cleave in the product amplified in the *vtc1-1* mutants but not that from wild type plants.

DNA was isolated from greenhouse grown plants, one plant per line per plot. The Bernatzky and Tanksley (1986) protocol was adapted for rapid extraction of small quantities. Flower buds were harvested in liquid nitrogen and grinded in 330µl of a preheated (65°C) extraction solution (125µl extraction buffer (0.35 M Sorbitol. 100 mM Tris, 5 mM EDTA, pH 7.5 (HCl) together with 175 µl lysis buffer (200 mM Tris, 50 mM EDTA, 2 M NaCl, 2 % (w/v) cetyl-trimethyl-ammonium bromide) to which 30 µl sarkosyl (10% w/v)) was added. The mixture of crude plant material and extraction solution was incubated for 30 minutes at 65°C with occasional shaking. Hereafter a solution of 400µl chloroform/isoamyl alcohol (24:1 v/v) was added and vortexed. After centrifuging for 5 minutes at maximum speed in an eppendorf centrifuge the waterphase was transferred to a new tube. An equal amount of isopropanol was added DNA was precipitated by carefully inverting the tube. After 10 min centrifugation at maximum speed in Eppendorf centrifuge the water-alcohol mixture was discarded and the pellet washed with 70% cold ethanol. The pellet was left to dry and dissolved in water containing RNAse A and incubated 30 minutes at 37°C, thereafter it was stored in the refrigerator

Table 2.3: Mutants used, their genetic background and reference

Mutant gene	Genetic background	Phenotype	Gene encoded/defect	Reference
fro1 (*frostbite1*)	C24	constitutive levels of ROS	NADH dehydrogenase subunit of mitochondrial respiratory complex	Lee et al., 2002
RD29A::LUC	C24	control for fro1	-	Lee et al., 2002
aba1-5	Col	ABA deficient	zeaxanthin epoxidase	Léon-Kloosterziel et al., 1996b; Rock and Zeevaart , 1991; Meyer et al., 1994
C3-7-1	Col	reduced dormancy	unknown	Raz, unpublished
etr1	Col	ethylene resistant	ethylene receptor with histidine kinase activity	Bleecker et al., 1988; Chang et al., 1993
jar1-1	Col	jasmonic acid resistant	adenylate forming enzyme	Staswick et al., 1992; Staswick et al., 2002
vtc1-1	Col	vitamin-C deficient	GDP-mannose pyrophosphorylase/ accumulates only 30% ascorbate	Conklin et al., 1996; Conklin et al., 1999
cad2-1	Col	cadmium sensitive	y-glutamyl-cysteine synthase/ accumulates only 15-30% glutathion	Howden et al., 1995; Cobbet et al., 1998
abi3-1	L*er*	abscisic acid insensitive3-1	B3 domain protein with B1 and B2 domain	Koornneef and Hanhart, 1984; Giraudat et al., 1992
abi3-5	L*er*	abscisic acid insensitive3-5	B3 domain protein with B1 and B2 domain	Ooms et al., 1993; Giraudat et al., 1992
abi3-7	L*er*	abscisic acid insensitive3-7	B3 domain protein with B1 and B2 domain	Bies-Ethève et al., 1999
ats	L*er*	aberrant testa shape	unknown/lacks 2 out of 5 integuments	Léon-Kloosterziel et al. 1994
gai	L*er*	gibberellin insensitive	GRAS transcription factor	Koornneef et al., 1985; Peng et al. 1997
rdo1	L*er*	reduced dormancy1	unknown	Léon-Kloosterziel et al., 1996a
rdo2	L*er*	reduced dormancy2	unknown	Léon-Kloosterziel et al., 1996a
rdo3	L*er*	reduced dormancy3	unknown	Peeters et al., 2002
tt4-1	L*er*	transparent testa 4-1	chalcone synthase deficient/ lacks brown tannins	Feinbaum and Ausubel, 1998
tt3-1	L*er*	transparent testa 3-1	dihydroflavenol-4-reductase/ lacks brown tannins	Shirley et al., 1995
ga1-3	L*er*	GA deficient	copalyl diphosphate synthase	Koornneef and van de Veen, 1980; Sun et al., 1992
cat1 cat3	Ws	catalase deficient	deletion of the catalase 1 and 3 sequence	Salomé and McClung, 2002

Table 2.4: Primers used to identify mutants with no obvious phenotype: name of the primer indicates the mutant which can be identified accordingly

primer name	primer 1 (5'> 3')	primer 2(5'> 3')	Ta (ºC)	endonuclease used	type
cat1	ccgagactctcagagatc	atcaaggatcgtgcgtctg	54		CAPS
vtc	cttgagaccattgactctcagga	gaggaccagcggtacctagtgg	60	BamH1	dCAPS
cad	aggtgacaagatcattggtc	caaacctataccagataagaac	54	Bsl1	CAPS

cat1 is used to identify the catalase mutant; vtc and cad primers are used to identify respectively the ascorbic acid (*vtc1-1*) and glutathione deficient (*cad2-1*) mutants. Ta: annealing temperature used for specific amplification, CAPS markers were amplified in 35 cycles of 30sec 94ºC, 30 sec Ta and 90 sec 72ºC. The vtc dCAPS was amplified in 35 cycles of 30sec 94ºC, 30 sec Ta and 60 sec 72ºC.

Culture and storage conditions

Seeds were sown in Petri dishes on water-saturated filter paper and incubated in the growth chamber at 25° C. After 2 days of incubation, germinated seeds were transferred to soil and cultivated in an air-conditioned greenhouse (18°- 23°C) in a 16 h photoperiod.

F_3 seeds of selected "double" mutant plants together with all controls were grown in 1 plot containing 4 randomized replicates of 6 plants. Seeds harvested, bulked from 5 plants, from mature dry siliques were 11 months after harvest and storage at ambient conditions, transferred to incubators containing saturated solutions of different salts $CaCl_2$ (20°C; 32% RH) and $CaNO_3$ (20°C; 60% RH). Seeds were left to age for 1 year in these incubators and sown on 0, 0.3 and 0.5% H_2O_2. Germination, of 40-80 seeds of three replicates, bulks of 5 plants, was scored after 5 days incubation at 4°C and subsequent incubation for 7 days in a growth chamber (25°C, 16 h light period).

The hormone and dormancy mutants were grown in 4 randomized replicas of 6 plants, seeds were harvested from mature dry siliques and bulked per replica and left in storage at ambient conditions. The L*er* seed lots were grown, and harvested, in the same year in the same greenhouse but in different experiments. Seeds from 4 replicates were sown in Petri dishes with $1x10^{-5}$ M GA_{4+7}, germination was scored after 5 days incubation at 4°C and subsequent incubation for 7 days in a growth chamber (25°C, 16 h light period). For statistical analysis germination data was probit transformed and analyzed with the statistical package SPSS version 11.0.1 (SPSS, inc, Chicago), p-values were adjusted for multiple comparisons within the same data set if necessary.

Seeds from the F_1 L*er* x *ats* and *ats* x L*er* together with the controls were harvested from mature dry siliques and stored at ambient conditions until use.

Controlled deterioration tests

Seeds that had been stored for either 9 months at ambient conditions (CDT1) or 9 months at ambient conditions and 2 months at 32% RH (CDT2) were used in a CDT. CDT was performed according Tesnier et al. (2002). Seeds are equilibrated at 85% relative humidity (15°C), 0 day controls are immediately dried back at 32% relative humidity. Treatment is done by storing the seeds (at 85% RH) for a number of days at 40°C (CDT 1 and CDT2, 1, 3, 5 and 7 days); after this also these seeds are dried back at 32% RH (20°C) and stored at circa 4°C until the germination was tested. Three replicates of 50 seeds were tested per line for each day of treatment. Final germination was determined after 14 days without (CDT1) or with (CDT2) cold treatment (7 days 4°C). Germination data of mutants and wild-types that did not show dormancy was estimated as a single parameter as the number of days of treatment required to reach 50% germination (D_{50}). Germination proportions of all treatments were used for probit regression on a time scale, in days, applying the regression module of the statistical package SPSS version 11.0.1 (SPSS, inc, Chicago); this was thereafter further analyzed, using the same program. The p-values were adjusted for multiple comparisons within the same data set.

Seeds of the F_1 L*er* x *ats* and *ats* x L*er* together with the controls were treated for 0,1,2 and 4 days at 40°C and 85%RH as described above. Two replicas of 60 to 100 seeds were sown without cold treatment and final germination was determined after 14 days. Statistical analysis was performed by transforming the germination data into probits and using the general linear model of the statistical software SPSS version 11.0.1 (SPSS, inc, Chicago).

Seeds of the *fro1* mutant and controls were harvested from mature dry siliques and stored under ambient conditions until use. CDT was performed with 3 replicas of 40 to 100 seeds, seeds were treated for 0 and 1 day at 40°C and 85% RH as described above, final germination was determined after 14 days.

Chapter 3

Characterization of *green seed*, an enhancer of *abi3-1* in Arabidopsis that affects seed longevity

Emile J.M. Clerkx, Hetty Blankestijn-De Vries, Gerda J. Ruys,
Steven P.C. Groot Maarten Koornneef

Abstract

Seeds are usually stored in physiological conditions in which they gradually loose their viability and vigor depending on storage conditions, storage time and genotype. Very little is known about the underlying genetics of seed storability and seed deterioration. We analyzed a mutant in *Arabidopsis thaliana* disturbed in seed storability. This mutant was isolated as a *grs* (*green seeded*) mutant in an *abi3-1* (*abscisic acid 3*) mutant background. Genetic and physiological characterization showed that the monogenic *grs* mutant was not visibly green seeded and mapped on chromosome 4. This enhancer mutation did not affect the ABA sensitivity of seed germination or seed dormancy but was found to affect seed storability and seedling vigor. Seed storability was assessed in a controlled deterioration test, in which the germination capacity of the mutant decreased with the duration of the treatment. The decrease in viability and vigor was confirmed by storing the seeds in two relative humidities (RHs) for a prolonged period. At 60% RH the mutant lost germinability but storage at 32% RH showed no decrease of germination although seed vigor decreased. The decrease in viability and vigor could be related to an increase in conductivity, suggesting membrane deterioration. This was not affected by light conditions during imbibition, expected to influence the generation of active oxygen species. During seed maturation ABI3 regulates several processes: acquiring dormancy and long-term storability and loss of chlorophyll. Our results indicate that GRS is a common regulator in the latter two but not of dorrmancy/germination.

This chapter has been published in Plant Physiology (2003) 132: 1077-1084

Introduction

Seeds of good quality are undamaged seeds that produce viable and vigorous seedlings without defects under various environmental conditions also after storage (Dickson, 1980). Depending on storage conditions, storage time and genotype seeds gradually loose vigor and viability. A common interpretation of the physiology of this seed deterioration cannot be given because the causes are assumed to be due to a variety of processes. For instance, mechanical damage that occurs in the field is different than physiological damage occurring during storage, which in itself may have different components (McDonald, 1999). Seed deterioration in agronomic crops is a problem that has been unintentionally aggravated by domestication because wild species often retain a high seed quality for many years (Moore and Halloin, 2000). Little is known about the genetic basis of differences in seed quality because this trait is affected by environmental effects during seed formation, harvest, and storage and is probably controlled by several genes and, therefore, is complex in genetic studies. To investigate the genetics of this process, one can identify mutations that either have an improved or a deteriorated seed quality (Tesnier et al., 2002). Among Arabidopsis mutants that have a poor seed quality, which is obvious by their rapid loss of viability upon storage, are mutants that affect seed maturation such as *lec1, lec2, fus3* (Holdsworth et al., 1999; Finkelstein et al., 2002) and strong *abi3* alleles (Nambara et al., 1992,Ooms et al., 1993). ABI3 is a B3 domain transcription factor that is assumed to control abscisic acid (ABA)-induced seed maturation and germination processes. Its effect on seed maturation is indicated by the reduced expression of many seed maturation-specific mRNAs (Parcy et al., 1994) and a reduced chlorophyll breakdown at the end of seed maturation in *abi3* null alleles (Nambara et al., 1995; Parcy et al., 1997). This results in the typical green seed phenotype of strong *abi3* alleles (Ooms et al, 1993). This green seed phenotype was also observed in double mutants that combined the weak *abi3-1* allele with ABA deficiency due to a mutation in the *ABA1* gene (Koornneef et al., 1989).

It is believed that the many, mainly seed specific, pleiotropic effects of *abi3* mutants may be because the ABI3 protein induces several pathways, which act independently after being promoted by the ABA/ABI3 system. However, some of these processes might be functionally related. For instance, it might be possible that the poor storability and the green seed phenotype have a causal relationship where the extra chlorophyll during imbibition may lead to photodamage (Thomas and Smart, 1993; Hendry, 1997). This is also suggested by the observation in cabbage (*Brassica oleracea*) seeds sorted based on their chlorophyll fluorescence, high amounts of chlorophyll correlated with lower quality in normal germination assays, but also in artificially deteriorated seeds (Jalink et al., 1998). It is also possible that both are processes that have functionally no relationship but have only in common that they

are both occurring during the later phases of seed maturation and have ABI3 as common inducer. When processes are independent, it might be possible to identify mutations that affect only one of these specific downstream pathways of ABI3. Such mutants can be isolated as enhancers or suppressors of specific aspects of pleiotropic mutants such as *abi3*. When such mutants modify all aspects of the mutant phenotype, it is expected that they directly influence the functioning of the pleiotropic gene. However, when only a subset of the phenotype is affected these modifiers may control specific processes downstream of the common regulator. Leaky mutant alleles provide good opportunities to find enhancers of specific mutant traits because they represent a sensitive background in which minor changes are easily observed (Page and Grossniklaus, 2002). In the present report, we describe a mutation that enhances the phenotype of the leaky *abi3-1* allele and show that this affects only a subset of the ABI3-induced processes, including aspects of seed quality.

Results

The genetic characterization of the green seed (*grs*) mutant

A mutant line with mature seeds that are greener compared with the parental genotype was isolated in the selfed progeny of ethyl methanesulphonate-treated seeds, carrying the leaky *abi3-1* allele. This phenotype, with a green embryo and a brown testa, resembled *abi3* alleles stronger than *abi3-1,* such as *abi3*-7 and null alleles of *ABI3*, which have been isolated in the same mutagenesis experiment (Ooms et al., 1993; Bies-Etheve et al., 1999). The analysis of the progeny derived from the cross of this mutant with the Landsberg *erecta* wild-type showed that only the seeds harvested on five out of 168 F_2 plants had a phenotype similar to that of the original green seed mutant. This ratio, which is not in conflict with a 1:15 ratio (χ^2= 3.07, P> 0.05), together with the observation that the green seed phenotype did not occur in seeds harvested on F_2 plants that were wild-type for the *ABI3* locus, shows that the green seed phenotype is only expressed in homozygous *abi3-1* mutants and inherits as a recessive mutation independently from *abi3*. This mutation can be considered as an enhancer of *abi3-1* because it leads to a phenotype resembling strong *abi3* alleles. This enhancer locus is called *grs*. Lines homozygous for the *grs* mutation in WT (non-*abi3-1* background) were identified as follows. Seeds harvested on F_2 plants that showed approximately 25% green seeds and that also segregated 25% of germinating seeds on 10 µM ABA were assumed to be homozygous for *grs* and heterozygous for *abi3-1* genotypes. F_3 plants from such progeny were screened for plants of which the F_4 seeds were fully sensitive to the germination-inhibiting effect of ABA. These single *grs* mutants did not differ phenotypically from wild-type plants, and their seeds had a normal brown wild-type color with a white mature embryo.

Linkage analysis using the progeny of an F_2 individual homozygous for *abi3-1* but segregating for green seeds showed that the *GRS* locus is located on chromosome 4 near *DET1* (Fig. 3.1), at a position where no other mutants of seed development have been located so far (Meinke et al., 2003).

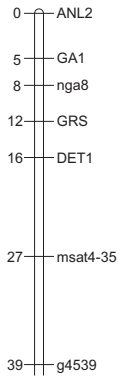

Figure 3.1. Map position of the *grs* mutation on chromosome 4.

Physiological characterization of the *grs* mutant.

A further characterization of the genotypes homozygous for *grs*, either with and without the *abi3-1* mutation, was performed to see if the *grs* mutation affects all aspects of the *abi3* mutant phenotype and if it has a recognizable phenotype without the *abi3-1* mutation. The four major characteristics of *abi3* mutations that we analyzed are: the sensitivity to applied ABA, seed dormancy, chlorophyll content in seeds, and seed quality. The reduced germination of seeds upon storage in adverse conditions and the number of abnormal seedlings were used as criteria for seed quality. The ABA sensitivity of all four lines was tested and showed that the single *grs* mutant is as sensitive to ABA as the wild-type of which complete inhibition of germination occurs at 50 µM, whereas the inhibition of the *abi3-1* and the *abi3-1 grs* double mutant is also similar (Fig. 3.2). This indicates that the *grs* mutation does not alter the response to ABA. In contrast to seed germination on ABA, small differences were observed in the dormancy of seeds (Fig. 3.3). Lines with the *abi3-1* mutation are much less dormant than genotypes with the wild-type *ABI3* allele as shown before (Bies-Ethève et al., 1999). Double mutants (*abi3-1 grs*) were slightly less dormant, indicating some enhancement of the *abi3-1* phenotype. However, the single *grs* mutant was slightly more dormant than the wild-type Landsberg *erecta,* which is opposite to the effect of this mutation in the *abi3-1* mutant background. We conclude that the *grs* mutation does not consistently affect seed dormancy.

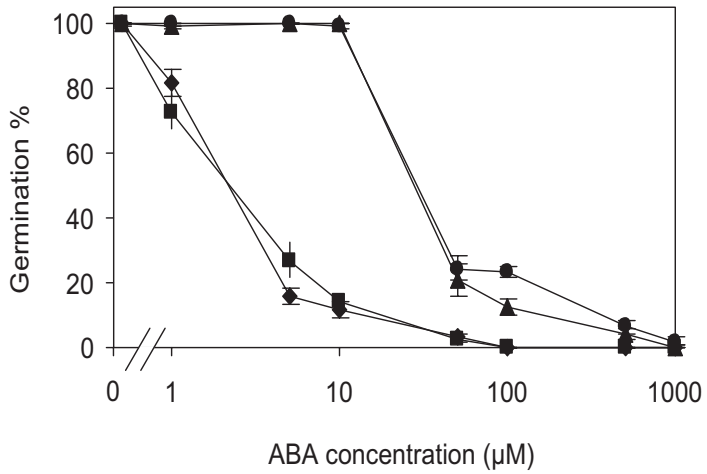

Figure 3.2. Germination in the presence of ABA of wild-type (diamonds), *abi3-1 GRS* (triangles) *ABI3 grs* (squares) and *abi3-1 grs* (circles). Data are averages of six replicates ± SE.

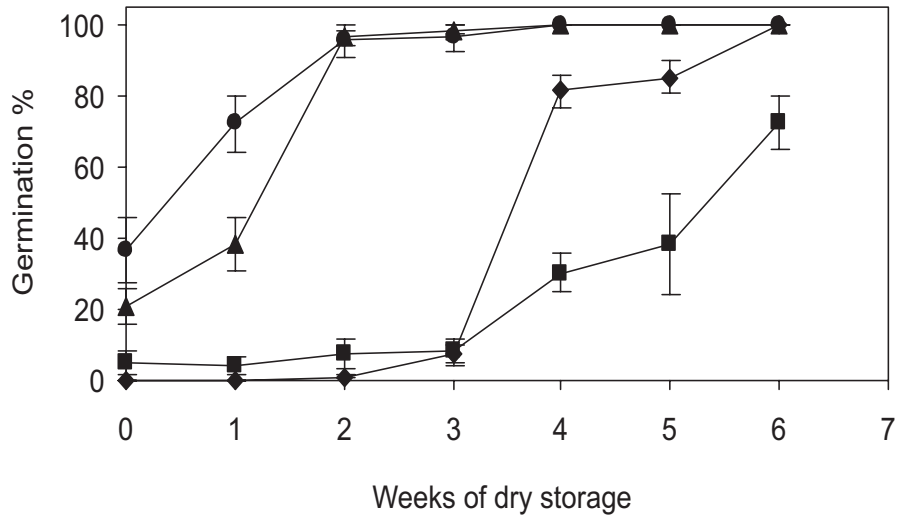

Figure 3.3. Germination behavior of seeds, in light harvested 22 days after flowering from mature dry siliques and subsequent dry storage till six weeks. Diamonds, wild-type; triangles, *abi3-1 GRS*; squares, *ABI3 grs*; circles, *abi3-1 grs*. Data are averages of six replicates ± SE.

The effect on which the selection of this mutant was based is the green color of its seeds. Chlorophyll measurements in the double mutant confirmed the expected higher chlorophyll content in the double mutant compared to the other three genotypes tested (Fig. 3.4). Although statistically not significant and not obvious by visual inspection, *abi3-1* seeds also seem to contain slightly more chlorophyll than wild-type, as has been shown before by Parcy et al. (1997). A slight increase in chlorophyll content is also suggested for the single *grs* mutant. Jalink et al. (1998) also noticed that cabbage seeds of different maturity that have the same color to the human eye can contain different amounts of chlorophyll.

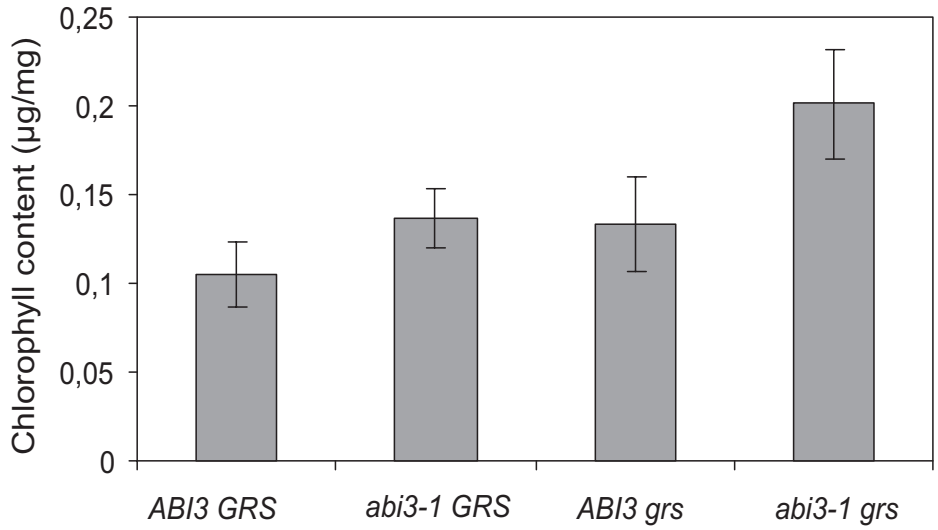

Figure 3.4. Analysis of total chlorophyll content (µg/mg seed) of wild-type (*ABI3 GRS*), *abi3-1 GRS, ABI3 grs* and *abi3-1 grs* seeds. Data are averages of 12 replicated extractions ± SE.

The reduced breakdown of chlorophyll might be an indication that seed maturation is not completed properly in the *grs* mutant, this effect could be additive to a similar effect in *abi3-1* and might lead to reduced desiccation tolerance of the seeds that is also observed in the *abi3* allelic series. Although only null alleles of *abi3* such as *abi3-4*, *abi3-5* and *abi3-6* cannot survive more than a few weeks of dry storage at room temperature (Nambara et al., 1992; Ooms et al., 1993), leaky *abi3* alleles such as *abi3-7* show this reduced storability when seed deterioration is enhanced in controlled deterioration (CD) tests as described for Arabidopsis by Tesnier et al. (2002). Therefore, we compared seed storability both in CD test conditions and under normal but less favorable conditions when seeds are stored at relative high humidity.

When seeds were stored at 60% RH and ambient temperature, viability was lost more rapidly in the double mutants than in the single mutants, which lost viability at a slower speed (Fig 3.5C). Even after 22 months of storage at 60% RH, the wild-type seeds still germinated almost 100%. The effect of storage at low humidity on preventing viability loss is shown by the absence of viability loss for all genotypes when seeds were stored for the same period at 32% RH (Fig 3.5A). In the germination assay done after 22 months of storage, the number of abnormal seedlings as percentage of total germination also was determined. It appeared that although storage at 32% RH did not show a large decline in total germination in the four genotypes, the double mutant produced the highest number of abnormal seedlings (Fig 3.5B). The effect is even more dramatic after storage at 60% RH (Fig 3.5D), where almost all the double mutant seeds that had germinated gave rise to seedlings with an aberrant phenotype.

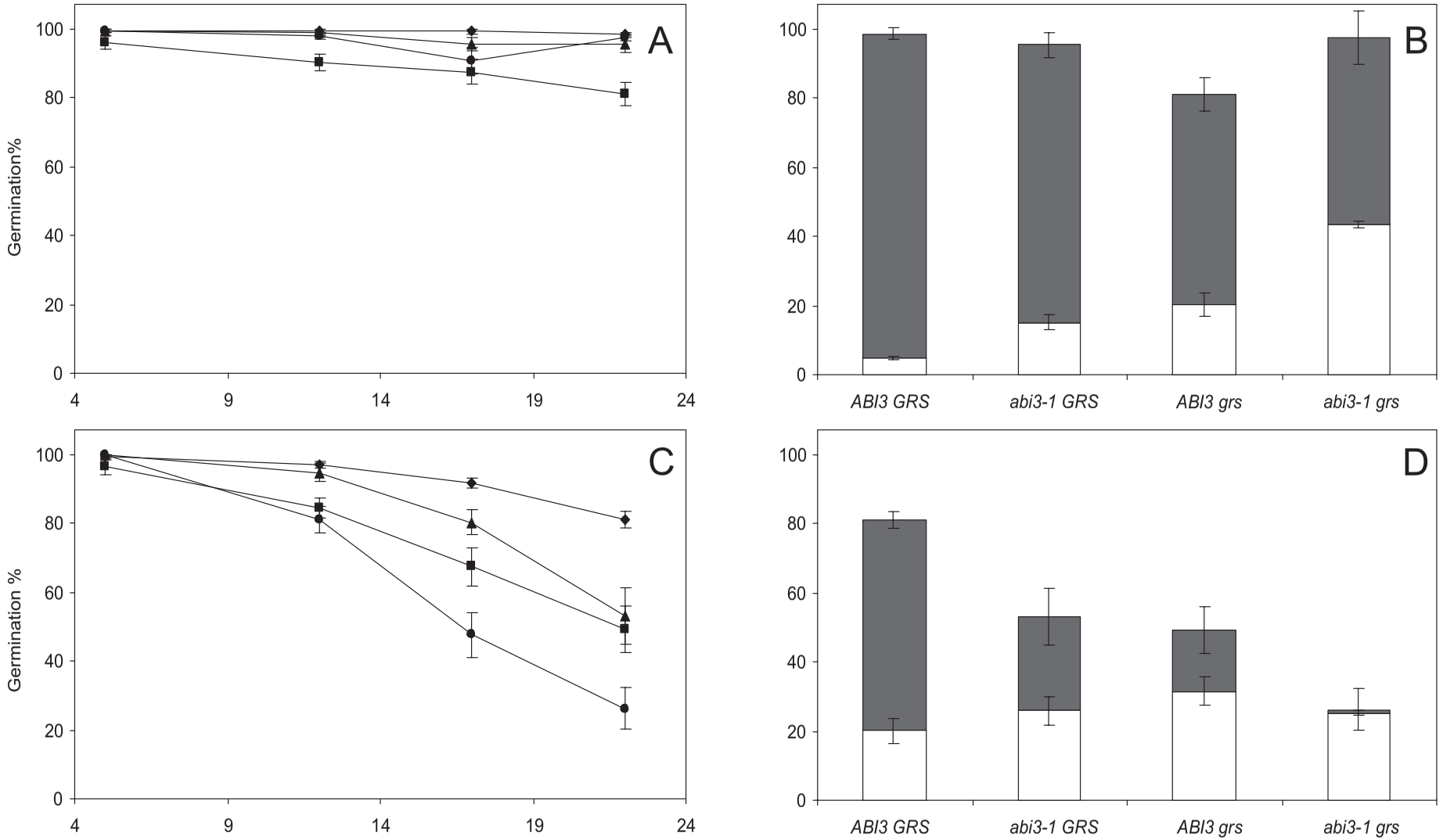

Figure 3.5. Seeds were stored at two different conditions, optimal 32% RH (A and B) and sub-optimal 60% (C and D). Germination behavior was tested during several time points during prolonged storage (A and C) of wild-type (diamonds), *abi3-1 GRS* (triangles) *ABI3 grs* (squares) and *abi3-1 grs* (circles). Bars (B and D) = total germination after 22 months of storage. In each bar the dark-gray part of the bar indicates the normal seedlings, and the white part represents the abnormal seedlings. Each point represents six replicates of 50-80 seeds ± SE.

In a CD test, simulating aging of seeds, seeds from the same batches and stored for 14 months at 32% (Fig 3.6A) or 60% RH (Fig 3.6B) were compared. The four genotypes showed viability loss in the order wild-type, single mutants, and double mutant, although in these conditions the *abi3-1* mutant seeds performed less well than the *grs* single mutants when seeds were stored at 32% RH. In seeds first stored at 60 % RH, even the wild-type is severely affected by the subsequent CD treatment, and the *abi3-1* mutant performs better then the single *grs* mutant. The additive effect of *abi3-1* and *grs* is obvious in all test conditions

Damage during dry storage of seeds that may result in loss of viability has often been associated with damage to membranes and may result in leakage of electrolytes. The amount of electrolytes released from imbibed seeds and measured as conductivity can be used as a method to assess quality of seeds (Illipronti, 1997). In the present experiment where both genetic and environmental factors affect the loss of viability we observed a negative correlation (r = - 0.74) between both parameters when results from both storage treatments were combined (Fig. 3.7). However, this correlation was absent when the genotypes were compared that were stored at 32% RH.

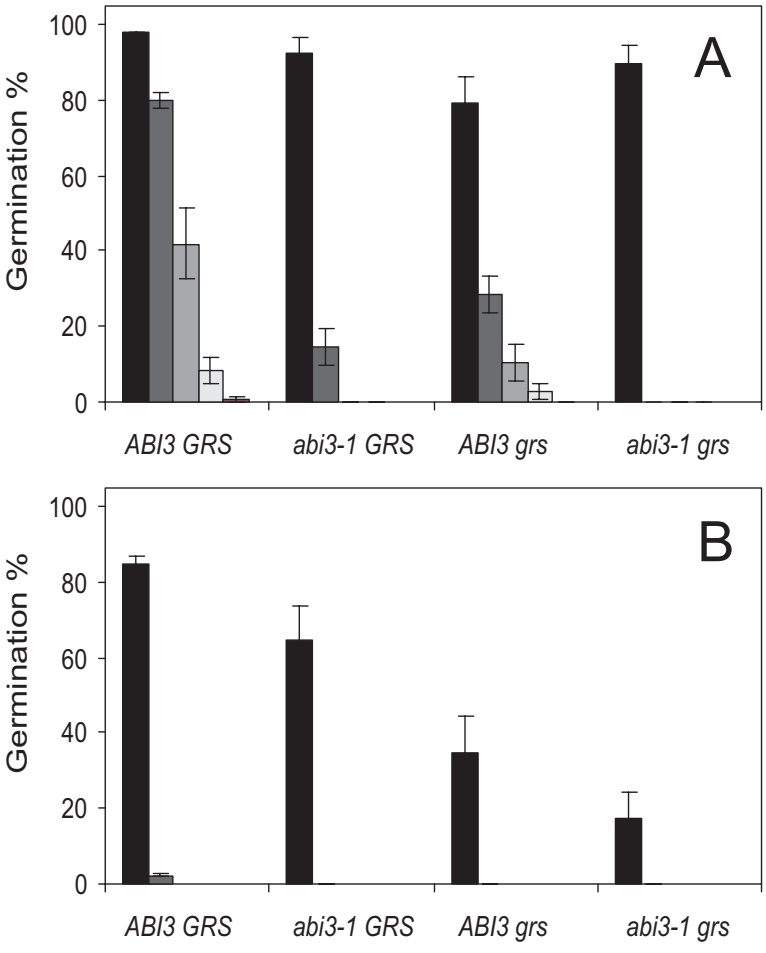

Figure 3.6. Effect of a CD treatment on wild-type (*ABI3 GRS*), *abi3-1 GRS*, *ABI3 grs* and *abi3-1 grs* seeds that had first been stored for 14 months in: A, optimal: 32% RH conditions; and B, suboptimal, 60% RH conditions. Both sets were treated for 1 (dark-gray bars), 2 (gray bars), 3 (light-gray bars), and 5 (white bars) d at 40°C and 85% RH. Control seeds (black bars) were only equilibrated at 85% RH. After this treatment, all seeds were dried back to 32% RH until sowing. Each bar represents a sowing of 3 replicates of 90 to 100 seeds ± SE.

Higher chlorophyll amounts could increase the amount of reactive oxygen species in light (Heath and Packer, 1968). When this would be the main source of damage, more damage should be expected upon imbibition in light compared with germination in darkness. Seeds that had been stored for 19 months at 60% RH were compared for germination in light respectively, in darkness (Fig. 3.8). The dark germinated seeds obtained only a 5-h light period to induce maximum germination. Again, differences were observed between different lines in total germination, but no significant differences could be observed due to differences in light conditions during germination.

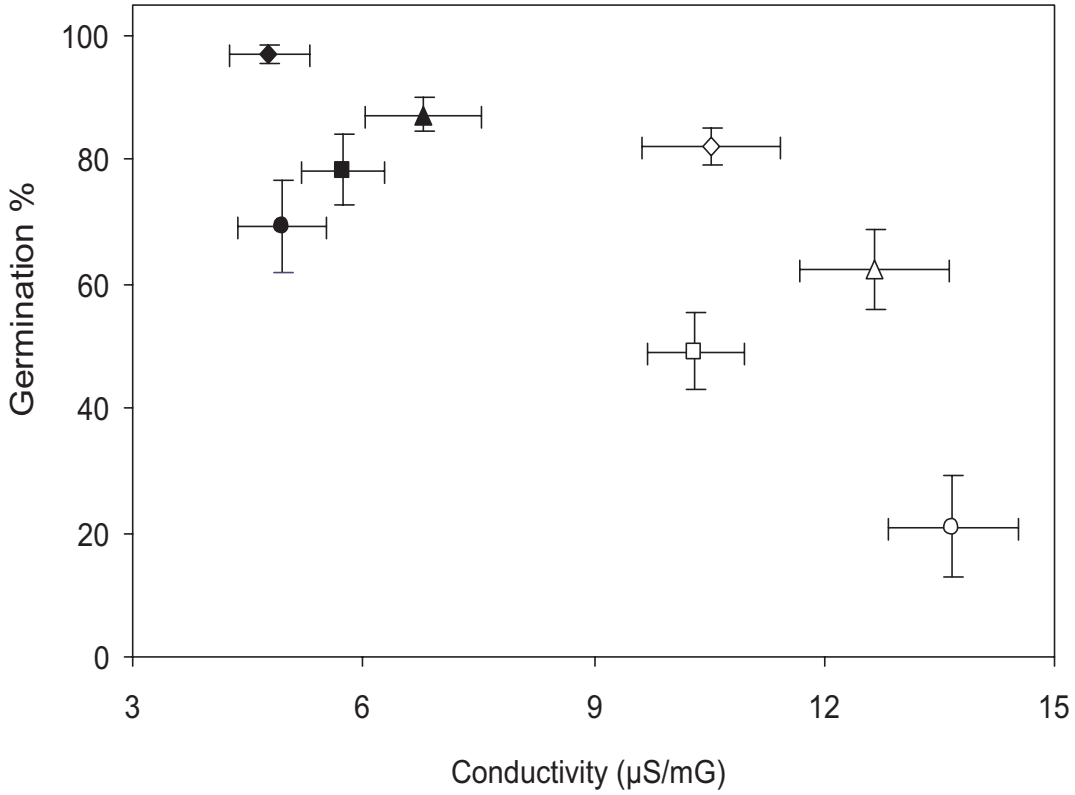

Figure 3.7. Conductivity measurement on seeds that were submerged in demiwater. The scatter diagram shows the effect of prolonged storage (19 months) on wild-type (*ABI3 GRS*, diamonds), *abi3-1 GRS* (triangles), *ABI3 grs* (squares), and *abi3-1 grs* (circles) under two conditions: favorable, 32% RH (black symbols); and unfavorable, 60% RH (white symbols), on germination and the conductivity after 24 h of imbibition. The germination assay was done with seeds from the same batches and age after a 7-day cold treatment, six replicates ± SE. Conductivity was measured in three replicates of bulked seeds ± SE.

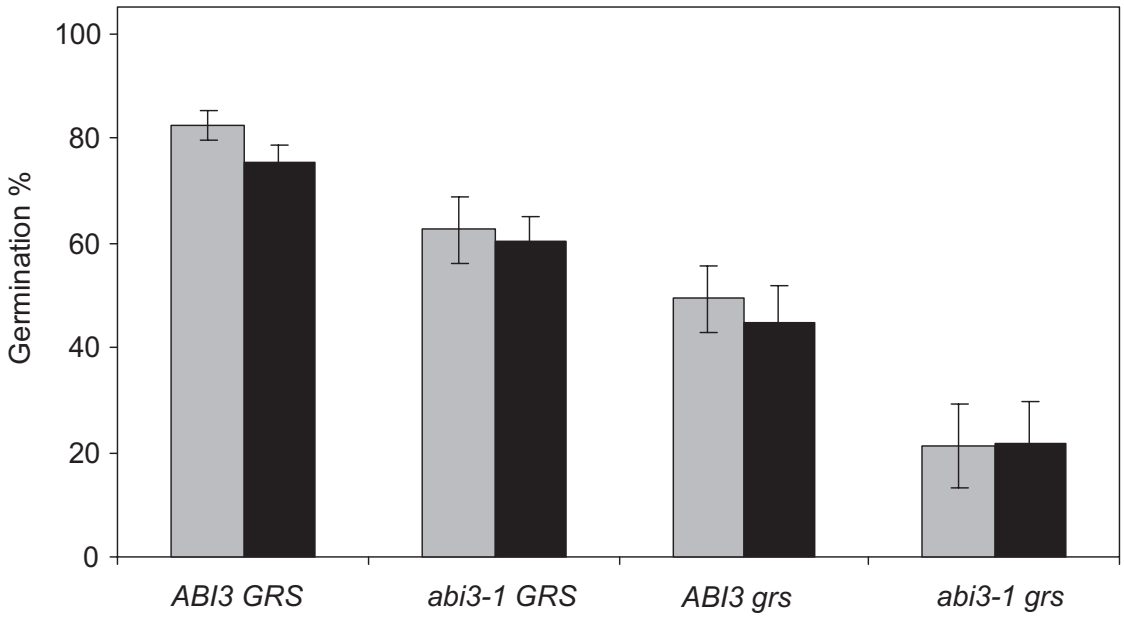

Figure 3.8. Germination of wild-type (*ABI3 GRS*), *abi3-1 GRS*, *ABI3 grs* and *abi3-1 grs* seeds stored 19 months at 60% RH. Maximal germination was induced by storing the seeds for 7 d in the cold before exposing the seeds to white light for 5 h (white light between 55-62 µmol m^{-2} s^{-1}). Thereafter, seeds for the dark treatment (black bars) were transferred to darkness for 7 d or kept in the light (gray bars) for 7 d, after which the germination percentage was recorded. Each bar represents six replicates ± SE.

Discussion

The plant hormone ABA affects a wide range of processes in plants. Examples are: altered gene expression, tolerance to cold stress, inhibition of growth, and tissue-specific stress responses like the closure of stomata. Furthermore, the plant hormone plays a role in seed maturation and dormancy. The various mutants used to elucidate the role of ABA in these processes have been reviewed by Leung and Giraudat (1998), Koornneef et al. (1998), and Finkelstein et al. (2002). The many pleiotropic effects of the *abi3* mutants indicate that this protein affects many processes during seed maturation and germination, including the storability of seeds. Storability under various conditions is an important aspect of seed quality. In this report, we describe the *grs* mutation that enhances some, but not all, aspects of the pleiotropic *abi3* mutant phenotype. The observation that ABA sensitivity is not affected and that not all ABA-controlled processes such as seed dormancy and stomata closure seem affected indicates that this enhancer locus does not control ABA metabolism or the mode of action of ABA in general. For instance, this is observed in the *aba1* mutant, which is also an enhancer of *abi3-1* (Koornneef et al., 1989). Therefore, we conclude that the *GRS* locus promotes only ABI3 induced degradation of chlorophyll and the storability of seeds. Although the effects on the latter are mild, they are observed in different storage conditions that are relatively unfavorable for maintaining seed quality. Therefore, the *grs* mutant can be described as a mutant specifically affected in seed quality, which is part of an ABI3-induced pathway that protects seeds from deterioration in a dry state. Because this mutant also affects chlorophyll degradation, especially in *abi3-1* background, it cannot be excluded that the maintenance of a high chlorophyll content leads to this reduced seed quality.

The loss of chlorophyll as seeds mature is referred to as degreening. Papers by Johnson-Flanagan et al. (1994) and Ward et al. (1995) describe problems with green seeds in relation to canola (*Brassica napus*) seed oil where this residual amount of chlorophyll promotes oxidation of the oil leading to rancidity. This chlorophyll content is usually due to immature seeds present in a harvest (Johnson-Flanagan and Thiagarajah, 1990; Ward et al., 1995). Immature seeds are known to have a reduced viability and vigor as was shown by Bailly et al. (2001),as the number of normal seedlings increased as the seeds matured. Steckel et al. (1989) showed that the germination performance of carrot (*Daucus carota*) seeds improved with decreasing amounts of chlorophyll and viability increased as seeds matured. The same negative relationship could also be found for geranium and soybean (*Glycine max*) seeds (Kwong, 1991; Illipronti, 1997). In one seed lot of cabbage seeds that was sorted based on their chlorophyll fluorescence and tested for their seed quality, high amounts of chlorophyll, as measured by chlorophyll fluorescence, correlated with lower quality in normal germination assays and in artificially deteriorated seeds (Jalink et al., 1998).

Senescence of the *grs* mutant fruits occurs at the same time after pollination as in wild-type control plants (data not shown) but this does not exclude the possibility of a reduced speed of seed maturation of the *grs* mutant within a normally maturing silique. It also cannot be excluded that the *grs* mutation affects specifically chlorophyll degradation, normally occurring at the end of the seed maturation process. A loss of chlorophyll breakdown is also described in many plants as a “stay green” phenotype as reviewed by Thomas and Smart (1993) in which they also describe some crop plants that display this phenotype in their seeds. Chlorophyll breakdown in leaves occurs in several sequential steps: hydrolysis of chlorophyll into chlorophyllide and phytol by the action of chlorophyllase; removal of Mg^{2+} from chlorophyllide to yield phaeophorbide a by Mg-dechelatase, cleavage of phaeophorbide a by phaeorphorbide a oxygenase (PaO) to get red chlorophyll catabolite (RCC), which in turn is reduced by RCC reductase. The final product is then transported over the tonoplast (Thomas et al., 2002). A number of enzymes involved in the breakdown of chlorophyll have been cloned in Arabidopsis: chlorophyllase (Tsuchiya et al., 1999), RCC-reductase (Wüthrich et al., 2000) and the ABC-type tonoplast transporter (Lu et al., 1998). However, none of them maps to the position found for *grs*.

The increased damage in the *grs* and *abi3-1* mutant and especially their double mutant could be related to their higher chlorophyll content because photosynthesis is a source of active oxygen species (Thomas and Smart, 1993; Hendry, 1997) that can damage biological systems. The problem could be 2-fold, the first one being an increase in active oxygen during storage decreased the seed quality, which could be possible because certainly for the storage at a higher RH, it is unlikely that all metabolic processes are stopped. Heath and Packer (1968) showed that light is required to generate radicals in chloroplasts and to prevent this effect, seeds were stored in exsiccators in darkness. Only upon sowing were seeds exposed to light for a short while. The second possibility is a lack of repair during the imbibition process; this would also be more damaging in light than in darkness. However, when seeds stored for 19 months at 60% RH were compared for germination in light or in darkness, no significant differences were observed, suggesting that imbibition in light does not increase viability loss.

Therefore, our physiological experiments do not suggest a causal relationship between chlorophyll retention and storability defects. Genetic studies with other species also fail to support a causal relationship. Mutants that retain chlorophyll in mature seeds have been described for soybean, but these *d1d2* and *cyt-G1* mutants have no impaired desiccation tolerance (Chao et al., 1995) such as the green seeded severe *abi3* alleles (Nambara et al., 1995) and *abi3-1 aba1* double mutants (Koornneef et al., 1989). Furthermore, Mendel’s peas (*Pisum sativum*), disturbed in the enzyme pheaorphorbide a oxygenase and, therefore, remaining green (Thomas et al., 1996), have not been reported as

having more problems with longevity compared to the yellow-seeded peas. Furthermore, desiccation intolerance can occur together with chlorophyll degradation, because the *lec* (*leafy cotyledon*) and *fus3* (*fusca*) mutants (Meinke et al., 1994) do not acquire desiccation tolerance but degrade their chlorophyll during seed maturation. ABI3 regulates several processes in maturation such as acquiring dormancy and long-term storability (desiccation tolerance) and chlorophyll breakdown. All these processes could be sequential processes. The existence of among others *rdo* (*reduced dormancy*) and *dog* (*delay of germination*) mutants, not green and desiccation tolerant (Léon-Kloosterziel et al., 1996a; Bentsink, 2002; Peeters et al., 2002) shows that acquiring dormancy can be separated from the other two. Therefore, we hypothesize that the green seed mutant described here is disturbed in a common step before chlorophyll breakdown and acquiring desiccation tolerance.

Both the *grs* and the *abi3-1* mutations resulted in seeds that exhibited increased electrolyte leakage upon imbibition after storage, and the effects of the mutations are additive. Increased leakage of electrolytes indicates a problem with cellular membrane integrity. High levels of seed electrolyte leakage can be because of a rapid inrush of water due to a high water potential gradient between dry seeds and the imbibition medium (Pandey, 1992) or to the damage caused by radicals either generated by excited chlorophyll or in the mitochondria. The first reason was prevented by pre-incubation of the seeds at 100% RH overnight. The second cause is unlikely because germination in light and darkness showed no difference. The last reason cannot be excluded, and McDonald (1999) suggests it as one of the causes for poor germination of aged seeds. The direct relation between the function of the wild-type *ABI3* and *GRS* genes in protection of the membranes during seed storage remains to be elucidated.

CD tests simulate aging of seeds under controlled conditions and can be used to predict seed storage potential (Hampton and TeKrony, 1995). Our results here confirm this because the double mutant that showed a decrease of germination during storage at 60% RH and an increase in the number of abnormal seedlings upon storage under favorable (32% RH) conditions also showed the largest response to the CD treatment. This is in agreement with results described by Tesnier et al. (2002), who indicated that differences in the same CD treatments can be due to genotype and not to variation in the treatment itself.

Materials and methods

Genotypes

Four genotypes were used in all experiments wild-type: Landsberg *erecta* (*ABI3 ABI3 GRS GRS*) *abi3-1* mutant (*abi3-1 abi3-1 GRS GRS*), the new single mutant, *ABI3 ABI3 grs grs* (brown seeded), and the double mutant *abi3-1 abi3-1 grs grs* (green seeded).

Culture and storage conditions

Seeds were sown in Petri dishes on water saturated filter paper and incubated in the growth chamber at 25°C. After 2 days of incubation, germinated seeds were transferred into soil and cultivated in an air-conditioned greenhouse (18°- 23°C) in a 16-h photoperiod. Plants were grown in two plots each with three randomized replicates of 12 plants per genotype. Eight plants were harvested in bulk per replicate for physiological analysis. Seeds harvested from mature dry siliques were stored in darkness 2 months after harvest in incubators containing saturated solutions of different salts $CaCl_2$ (20°C; 32% RH) and $Ca(NO_3)$ (20°C; 60% RH). Seeds were left to age and sown 5, 12, 17 and 22 months after harvest. Germination was scored after 7 d of incubation in a growth chamber (25°C, 16 h light period). With the germination test performed after storage for 22 months, the abnormal seedlings were also counted: seedlings with an altered root growth, dwarfed, not fully unfolded cotyledons (cotyledons trapped in remains of the seed coat) or those that showed yellow or necrotic lesions on cotyledons, were considered abnormal.

Mapping

To map the *grs* locus, a mapping population was made by crossing the *abi3-1 abi3-1 grs grs* line to Columbia. An F_2 of 400 plants was generated, and F_3 seeds were harvested per F_2 plant. By germinating these F_3 seeds on 10µM ABA, the *abi3-1* homozygote plants were selected (approximately 25%). Only the DNA of these *abi3* mutant plants was used for molecular marker analysis, and the green seed phenotype was determined by eye in all F_3 lines. When there was any doubt about the phenotype, a next generation was grown, and the F_3 seed phenotype was determined based on the F_4 progeny. The map was created with the program Joinmap (version 3.0, Plant Research International, Wageningen, The Netherlands) and is based on 88 individuals.

DNA isolation and PCR conditions

DNA was isolated from greenhouse-grown plants. The Bernatzky and Tanksley (1986) protocol was adapted for rapid extraction of small quantities. Flower buds were harvested in liquid nitrogen and grinded in 330µL of a preheated (65°C) extraction solution (125µL of extraction buffer 0.35 M sorbitol. 100 mM Tris, 5 mM EDTA, [pH 7.5] together with 175 µL lysis buffer (200 mM Tris, 50 mM EDTA, 2 M

NaCl, 2 % [w/v] cetyl-trimethyl-ammonium bromide) to which 30 µL of 10% (w/v) Sarkosyl was added. The mixture of crude plant material and extraction solution was incubated for 30 minutes at 65°C during which period occasional shaking was applied. Hereafter, a solution of 400 µL chloroform:isoamyl alcohol (24:1 [v/v]) was added and vortexed. After centrifuging for 5 min at maximum speed in an Eppendorf centrifuge (Eppendorf Scientific, Westbury, NY), the water phase was transferred to a new tube. An equal amount of isopropanol was added to the tube and to precipitate the DNA it was inverted carefully several times. After 10 min centrifugation at maximum speed in an Eppendorf centrifuge, the water-alcohol mixture was discarded and the pellet washed with 70% (v/v) cold ethanol. The pellet was left to dry and dissolved in water containing RNAse A and incubated 30 minutes at 37°C; thereafter, it was stored in the refrigerator. The CAPS and microsatellite markers were either found in the The Arabidopsis Information Resource database (http://www.arabidopsis.org) or taken from http://www.inra.fr/qtlat/msat. The PCR conditions for the CAPS markers were adapted to the primer pair for the microsatellite markers; a standard protocol of 30s 94°C, 30s 50°C and 30s 72°C (35 cycles) was used.

Seed germination assays

To determine the sensitivity of germination to ABA, 40-80 seeds harvested from siliques of approximately the same age and all six replicates were sown on filter paper soaked with a range of ABA concentrations in 6-cm plastic Petri dishes. The imbibed seeds were stored for 4 d at 4°C and subsequently incubated in a growth chamber (25°C, 16 h light period). Germination was scored 7 d after the start of incubation at 25°C. For the dormancy release experiments, seeds were harvested from siliques of flowers that were tagged on the day of anthesis. Germination was scored by sowing 30-60 seeds harvested on each of the six replicates on Petri dishes containing filter paper soaked with water. Germination was scored after 7 d incubation in a growth chamber (25°C, 16 h light period); this was done six times with 1-week intervals. To compare germination in light and darkness, seeds from mature dry siliques, stored for 19 months at 60% RH as described before, were sown in the dark in two batches of six replicates and stored for 7 days at 4°C. Thereafter, they were placed in light for 5 h (55 - 62 µmol m^{-2} s^{-1}) to induce maximum germination; then, they were further imbibed in either darkness or 25°C, 16-h light period for 7 d when germination was determined.

Controlled deterioration tests

Seeds that had been stored for 14 months, as described before at 32% RH and 60% RH, were used in a CD assay. The CD assay (Tesnier et al., 2002) was performed as follows: seeds are equilibrated at 85% relative humidity (15°C), and 0-d controls are immediately dried back at 32% RH. Treatment is done by storing the seeds (at 85% RH) for a number of days at 40°C (1, 2, 3 and 5 d here); after this, these seeds were also dried back at 32% RH (20°C) and stored at approximately 4°C until the germination was tested. Four replicates of 90 to100 seeds were germinated on water-saturated thick filter paper placed on a “Copenhagen” germination table, at 25°C and with an 8-h light period per day.

To improve detection of radical protrusion, a black filter membrane (Schleicher and Schuell, Dassel, Germany, ref.-nr: 409712) was placed between the seeds and the filter paper. The seeds were covered with a transparent bell-shaped cover to prevent drying, and the final germination was evaluated 14 d after transferring the seeds to the “Copenhagen” germination table.

Chlorophyll extraction

All genotypes grown in one plot as six randomized replicates were harvested as mature dry seeds. Five milligrams was left to stand overnight in 200 µL dimethyl sulfoxide at 65°C, a second extraction was done in 150µL of dimethyl sulfoxide; therefore, in total, each genotype was measured 12 times. Absorption was measured at two wavelengths (649 nm and 665 nm) in a Beckman DU-64 spectrophotometer (Beckman Instruments, Fullerton, CA). Chlorophyll *a* content was calculated according to the following formula: (13.95 X A_{665})- (6.88 X A_{649}). Chlorophyll *b* was calculated according to the formula: (24.69 X A_{649})- (7.32 X A_{665}).

Conductivity measurement

Seeds from mature dry siliques were harvested and stored at 32% RH and 60% RH as described above. Nineteen months after harvest, 5 mg of two batches (1 from 32% RH and 1 from 60% RH) of three replicates were weighed. Germination of the two batches was scored by sowing them and incubating at 4°C for 7 d. Final germination was scored after 7 d at 25°C in 16-h light period. They were placed at 100% RH overnight to reduce imbibitional damage. After that, conductivity of all samples was measured in a CM100 conductivity meter (Reid & associates, Durban, South Africa).

Acknowledgements

We would like to thank Karin Léon-Kloosterziel for isolating the original mutant, Soazig Guyomarc’h for her help with the germination assays and the mapping, Dr. Folkert Hoekstra for the use of the conductivity meter, and colleagues at the Laboratories of Plant Physiology and Genetics and the Stichting Toegepaste Wetenschappen Supervision Committee for useful suggestions and discussions. This work was supported by the Technology Foundation STW, by the Applied Science Division of NOW, and by the Technology Program of the Ministry of Economic Affairs

Chapter 4

Selection of modifiers involved in seed storability in a "sensitized" genetic background

Emile J.M. Clerkx, Hetty Blankestijn-De Vries, Gerda J. Ruys,
Steven P.C. Groot. Maarten Koornneef

Abstract

Plants can survive unfavorable seasonal conditions as dry seeds. Desiccation tolerance and storability are essential and develop during seed maturation. Mutants in genes like *LEAFY COTELYDON1-3* (*LEC1-3*) and *ABSCISIC ACID INSENSITIVE3* (*ABI3*) that regulate seed maturation are characterized, among others, by desiccation defects and very reduced storability. Studying complex traits, like seed desiccation tolerance and storability by mutagenesis is complicated due to redundancy in genes and mechanisms affecting this process. Single mutants might have no, or very subtle, effects but may show a much more pronounced phenotype when they occur in a "sensitized" genetic background. We used the *abi3-5* mutant to find novel mutants affecting seed maturation or a specific subset of the maturation process by selecting for an enhanced storability. These mutations are suppressors of the *abi3* mutation and hence called *suppressor of abi* (*sua*). Four different mutants were isolated in an *abi3-5* sensitive background that consistently showed a much better germination after prolonged storage compared to *abi3-5*. In addition we used the genetic variation present among wild Arabidopsis accessions to identify modifiers of the *lec1-3* and *abi3-5* mutations. The selection in the "sensitized" background of *lec1-3* resulted in three lines with an improved storability and a similar selection in *abi3-5* resulted in five lines showing improvement. Map positions could be appointed to all new mutations and most modifiers isolated from different accessions. Furthermore the modifiers of *lec1-3* were reselected in their wild-type background based on the phenotype of improved mutant sister lines. No obvious storability phenotype could thus far be attributed to these reselected lines.

Introduction

Surviving unfavorable growth conditions is essential for plants that cannot escape adverse conditions by moving to better-suited environments. One way in which plants can survive such conditions is by their seeds, which can survive in dry state and often exhibit seed dormancy or metabolic quiescence. This will allow seeds, which are the most stress tolerant phase in a plant life cycle, to postpone further development even when imbibed (Farnsworth, 2000).

Stress tolerance in seeds develops during seed maturation, which is after morphogenesis of the embryo is completed (Koornneef et al., 1989; Bewley and Black, 1994). When seeds acquire stress tolerance a number of metabolic and molecular changes coincide, such as the transcription of late embryogenesis abundant (*LEA*) genes. In addition, orthodox seeds lose water, become desiccation tolerant and are metabolically quiescent (Baud et al., 2002). Acquiring desiccation tolerance might be considered as a prerequisite for seed longevity (Wilson Jr, 1995; Ingram and Bartels, 1996). Genes like *LEC1, LEC2, FUSCA3* (*FUS3*) and *ABI3* are the major regulators of seed maturation specific processes, like acquiring longevity and desiccation tolerance (Holdsworth et al., 1999; Finkelstein et al., 2002). Mutants of these genes will develop into normal looking plants, when rescued immaturely or shortly after harvest, indicating that these genes have seed maturation specific functions. The function of these genes is not fully overlapping as is inferred from their mutant phenotype, which especially distinguished the *LEC/FUS3* group from *ABI3*. However, differences between the *LEC1* and *FUS3* gene functions have also been described (Raz et al., 2001). The *lec1* mutant shows strong morphological alterations, already in the early events of seed maturation. Abnormalities are first detected at the torpedo stage during seed development (Meinke et al., 1994). Cotyledons of *lec1* mutant seeds accumulate anthocyanins primarily at the tip of the cotyledons (Meinke et al., 1992; West et al., 1994; Parcy et al. 1997). The cotyledons of *lec1* embryos carry trichomes and have a vascular system intermediate between that of cotyledons and leaves and embryos are occasionally viviparous at maturity. The *LEC1* gene encodes a transcription factor resembling a HAP3 subunit of a CCAAT box-binding protein (Lotan et al., 1998). *LEC1* does not act in isolation to control gene expression central in seed maturation programs, but in concert with other genes like *ABI3*, *LEC2* and *FUS3* (Parcy et al., 1997), which encode B3 proteins. Mutant null alleles of *ABI3* have a reduced expression of many seed maturation specific mRNAs and proteins and remain non-dormant (Ooms et al, 1993; Parcy et al., 1994). Different from the *lec* and *fus3* mutants is that mutant *abi3* seeds display a reduced chlorophyll breakdown at the end of seed maturation, resulting in the typical green seed phenotype (Nambara et al., 1995; Parcy et al., 1997) and that they are resistant to the germination inhibiting effects of abscisic

acid (ABA). Mutant *lec1* and *abi3-5* seeds have a strongly reduced storability, since these seeds loose their viability within a few weeks, whereas wild-type Arabidopsis seeds can be stored for many years at ambient conditions. In addition to these major regulators of seed maturation, other genes possibly including the target genes of the regulatory genes described above, could affect the development of desiccation tolerance and storability of seeds. Probably this concerns genes that act together in a redundant way, since it is striking that no other mutants, with major effects on desiccation tolerance of seeds, have been described in Arabidopsis.

The study of complex traits like seed storability and desiccation tolerance by mutagenesis is complicated by this redundancy. Only a combination of single mutants will reveal the role of these genes in a particular process. Single mutants might have no or very subtle effects, but may show a much more pronounced phenotype when they occur in a "sensitized" genetic background. A "sensitized" background can be described as a genotype already harboring a mutation in the process under study (Matin and Nadeau, 2001) and will allow the identification of so-called enhancer and suppressor mutants (Page and Grossniklaus, 2002).

Besides mutagenesis natural variation provides another source of genetic variation, although no large effect on seed vigor is expected since natural selection would have eliminated such genotypes. Effects of the genetic background on the expression of specific alleles of genes are often described as modifiers. Depending on the nature of the phenotypic effect, modifiers might cause more extreme (enhanced) phenotypes, less extreme (reduced, or even suppressed) phenotypes or novel (synthetic) phenotypes. Such modifiers are in fact the suppressors and enhancers that are present within the natural genetic variation of the species and can affect penetrance, dominance modification, expressivity and pleiotropy. Epistasis, which occurs when an allele of one gene masks the phenotype of another gene, is one of the genetic explanations for observing the effect of genetic modifiers (Nadeau, 2001).

In this study we used the *abi3-5* mutant, disturbed in late seed maturation, to find novel mutants affected in seed maturation or in a specific subset of the maturation process by selecting for enhanced seed storability. We were able to select four mutants, with enhanced storability, that are suppressors of the *abi3* mutation or some of the multiple processes, like ABA sensitivity, controlled by this gene. Furthermore we used the large genetic variation present among wild Arabidopsis accessions to identify modifiers of the *lec1-3* and *abi3-5* mutations. Selection provided in total eight modifiers prolonging the storability of *lec1-3* and *abi3-5*. When such genes affect structural elements of the desiccation tolerance/seed viability they may contribute to an effect that is not easily detected in wild-type background. Seed viability assays require long-term experiments or techniques in which seed deterioration is accelerated. To test if such modifiers affect seed vigor by itself we

separated the modifier genes, in the case of *lec1-3*, from this mutation and used these lines in a controlled deterioration (CDT) test developed for Arabidopsis by Tesnier et al. (2002)

Results

Selection of modifiers of *abi3-5* using mutagenesis

Mutagenesis with gamma rays was performed with a genetically marked *abi3-5 gl1 tt5-1* line to avoid that the selected suppressors of the *abi3-5* mutant were contaminated with wild-type seeds (see Materials and Methods). In total approximately 20000 seeds were mutagenized in two separate experiments. We selected for those seeds that germinated after eight weeks of dry storage. After three rounds of re-testing we selected four mutants that consistently showed a much better germination after storage. These mutants were named *suppressor of abi 1* to *4* (*sua*). Figure 4.1A shows the M_5 generation of *sua1*, *sua2* and *sua3* selected in the first experiment. Figure 4.1B shows *sua4*, which was isolated in the second mutagenesis experiment.

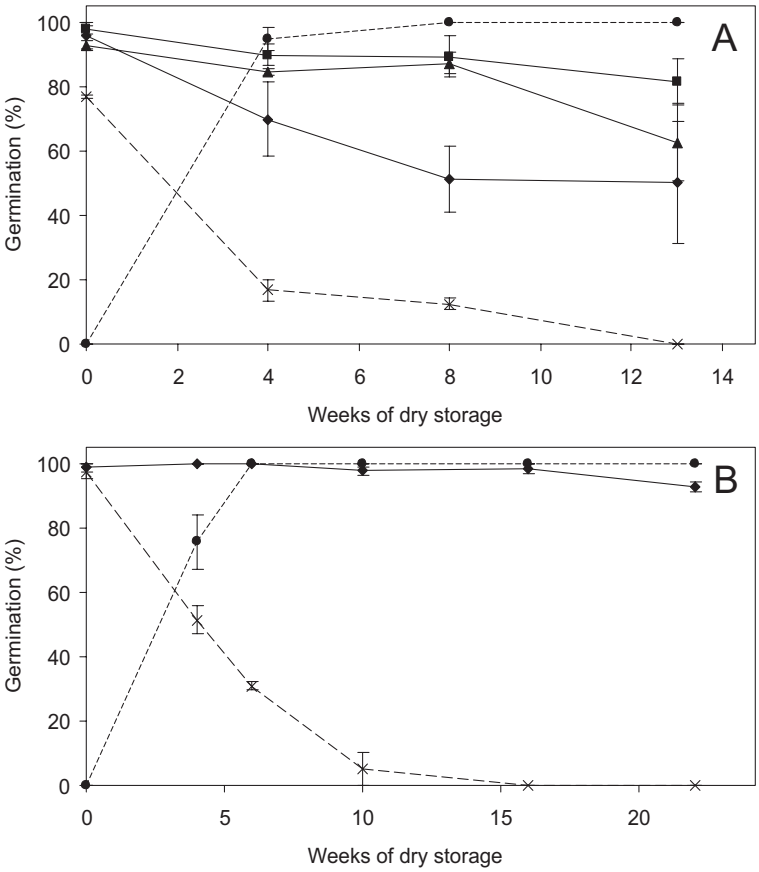

Figure 4.1. Germination behavior of isolated mutant line seeds harvested from mature siliques and subsequent dry storage; data are averages of three replicates of 40 to 80 seeds ± SE. A: a M_5 generation of *sua1* (diamonds), *sua2* (squares) and *sua3* (triangles) in *abi3-5 gl1 tt5-1* background are presented with the controls *abi3-5 gl1 tt5-1* (X and dashed line) and wild-type L*er* (circles and dashed). **B**: *sua4* (diamonds), M_4 generation, isolated in a second selection presented with the controls: wild-type (L*er*: circles and dashed) and *abi3-5 gl1 tt5-1* (X and dashed line).

Selection of modifiers of *abi3-5* and *lec3-1* mutants using natural variation

Twenty Arabidopsis accessions that had been chosen because they were relatively early flowering were crossed to *abi3-5 gl1 tt5-1* and *lec1-3*. The progeny of approximately 1200 F_2 plants with the *abi3-5* or *lec3-1* phenotype was tested for germination after 4 weeks of dry storage. Selected were those F_2 seeds of which their selfed progeny showed an enhanced storability. This selection of improved storability was confirmed in subsequent generations and resulted for the *abi3-5 gl1 tt5-1* mutants in the selection of five lines derived from crosses with five different accessions (Fig 4.2A). These are Gluckingen (*abi*/Gu), Seis am Schlern (*abi*/Sei), Warschau (*abi*/Wa), Eilenburg (*abi*/Eil) and Columbia (*abi*/Col). The selection in the "sensitized" background of *lec1-3* (ll M-1M-1 in Fig 4.3) resulted in three lines with an improved storability, two of which derived from Shakdara (*lec*/Sha1 and *lec*/Sha2) and one from Gluckingen (*lec*/Gu). Figure 4.2B shows the germination percentages of these three selected lines in the F_4 generation at different time points after harvest. This pattern of better storability was also monitored in later generations and proved to be consistent. In all cases the original mutant phenotypes, *abi3-5 gl1 tt5* seeds being green and *lec1-3* seeds containing anthocyanins, were still visible. When there was any doubt about the *abi3-5* and *lec1-3* mutant genotype, allele specific molecular markers for both mutations (see Materials and Methods) were used to confirm the genotype. The L*er* accession was also crossed to both mutants but this did not result in an improved storability as was expected since both mutants were in this genetic background.

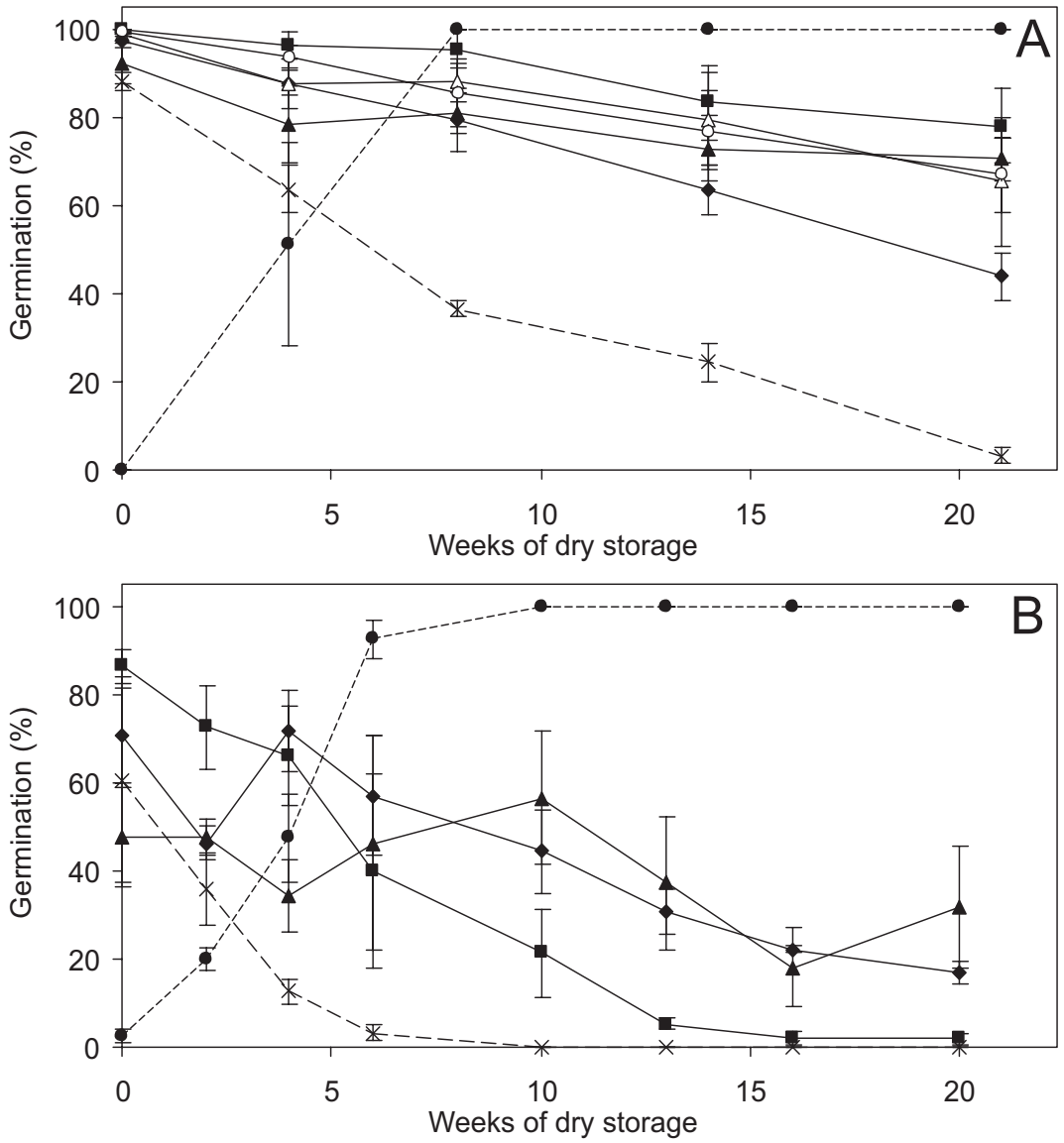

Figure 4.2. Germination behavior of selected modifier line seeds harvested from mature siliques and after subsequent dry storage, data are averages of three replicates of 40 to 80 seeds ± SE. No cold treatment was applied during the germination assay therefore the control L*er* shows a release of dormancy phenotype. **A**: F_5 generation of abi/Gu (closed squares), *abi*/Sei (closed triangles), *abi*/Wa (open circles), *abi*/Eil (open triangles), *abi*/Col (closed diamonds) in *abi3-5 gl1 tt5-1* mutant background are presented with the controls wild-type L*er* (closed circles and dashed) and *abi3-5 gl1 tt5-1* (X and dashed line). **B**: F_4 generation of *lec*/Sha1 (closed squares), *lec*/Sha2 (closed diamonds) and *lec*/Gu (closed triangles) in *lec1-3* mutant background are presented with the controls wild-type L*er* (closed circles and dashed) and *lec1-3* (X and dashed line).

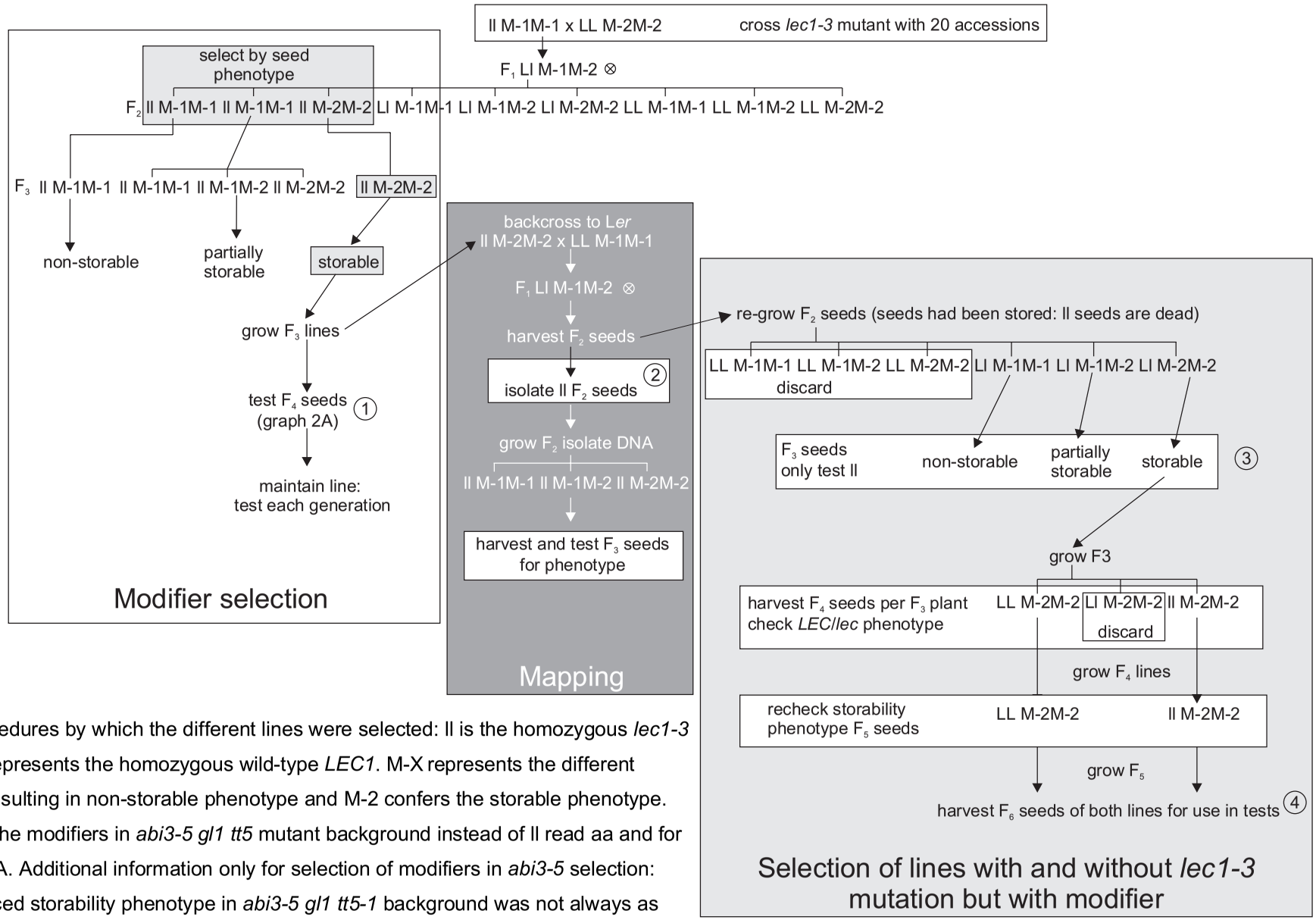

Figure 4.3. Procedures by which the different lines were selected: II is the homozygous *lec1-3* mutant and LL represents the homozygous wild-type *LEC1*. M-X represents the different modifiers: M-1 resulting in non-storable phenotype and M-2 confers the storable phenotype. For selection of the modifiers in *abi3-5 gl1 tt5* mutant background instead of II read aa and for wild-type read AA. Additional information only for selection of modifiers in *abi3-5* selection: since the enhanced storability phenotype in *abi3-5 gl1 tt5-1* background was not always as clear as in the *lec1-3* mutant background an extra round of selection was necessary (1 + 4). In *abi3*-5 modifier lines selection of heterozygous seed-lots was done on ABA after storage: storable lots had between 10 and 20% germination (2). Again due to the variable penetrance of the storability phenotype an extra round of selection was required (3).

Characterization of mutant and modifier lines

Our main selection criterion for both the mutant suppressor and natural modifier lines was an improved storability of the *abi3-5* and *lec1-3* mutants. During the selection of the natural modifiers we used both the phenotypic appearance of the *abi3-5 gl1 tt5* and the *lec1-3* mutants and their ability to readily germinate directly after harvest. This implies that only seeds in which the morphological changes were limited and having reduced dormancy, as seen in the original mutants, could have been isolated. In the mutant screen performed in homozygous *abi3-5 gl1 tt5* mutants, this bias was absent since the first selections were performed by storing the mutagenised seeds until the un-mutagenised control seeds had died. This leaves a possibility to isolate mutants that were altered for all traits affected in the *abi3-5* mutants including dormancy.

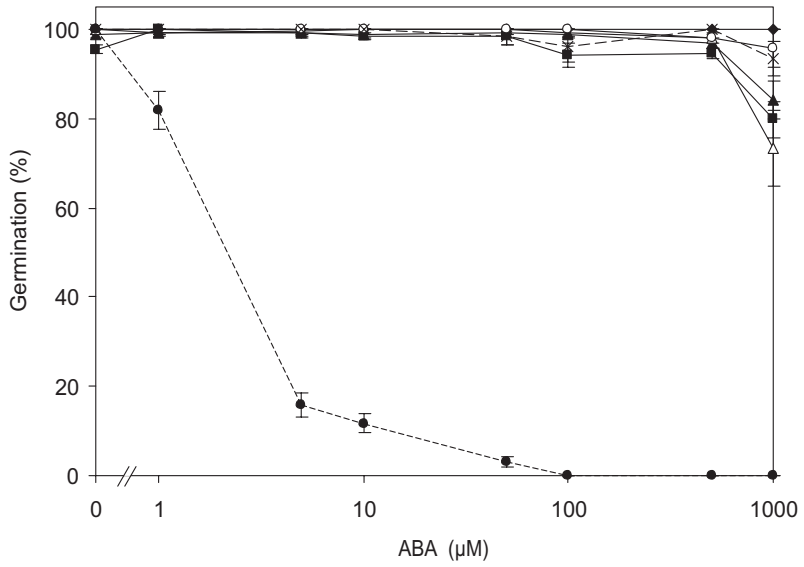

Figure 4.4. ABA-response curves of *abi*/Col (closed diamonds), *abi*/Wa (open circles), *abi*/Sei (closed triangles), *abi*/Gu (closed squares) and *abi*/Eil (open triangles) are shown with the controls wild-type L*er* (closed circles and dashed) and *abi3-5 gl1 tt5-1* (X and dashed line). The germination assay was done with F_8 seeds and a 6-day cold treatment; each point represents three replicates of 40 to 80 seeds ± SE.

As can be concluded from Fig 4.2A all selected *abi3-5* modifier lines and all suppressor mutants were still non-dormant. Figure 4.4 shows that only at the highest concentration of 1000 µM ABA a small inhibiting effect could be observed in *abi*/Sei, *abi*/Gu and *abi*/Eil, whereas the other two isolates were unaffected, indicating that ABA sensitivity had not changed. The green seed color of *abi3-5* was not fully penetrant in the *abi*/Eil, *abi*/Gu and *abi*/Wa lines; the color varied depending on the growth conditions. In *abi*/Col and *abi*/Sei the

green color was less variable and these seeds were greener, although some variation could also be observed depending on the season. The ABA response of the mutant modifiers (Fig 4.5) was unaltered in *sua3*. However the s*ua1* and *sua2* mutants showed a slight inhibition at higher ABA concentrations, whereas ABA resistance in *sua4* was clearly reduced. The latter mutant also showed a less obvious green seed color as did *sua3*. The *sua1, sua2* and *sua3* mutants showed elongated hypocotyls. In the progeny of backcrosses with L*er* this trait could be removed without losing the improved storability phenotype, indicating that this phenotype is not part of the pleiotropic phenotype of these mutations. The *sua2* mutant showed a somewhat darker green plant phenotype also in the selected backcross progeny, but further backcrossing will be necessary to see whether this phenotype is a pleiotropic effect of the *sua2* mutation or due to a second independent mutation

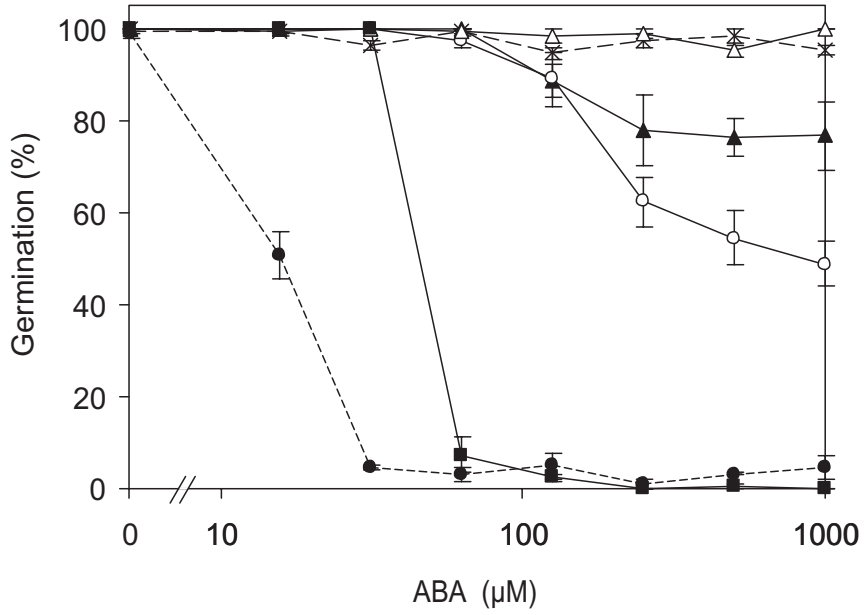

Figure 4.5. ABA-response curves of *sua1* (closed triangles), *sua2* (open circles), *sua3* (open triangles) and *sua4* (closed squares) are shown with the controls wild-type L*er* (closed circles and dashed) and *abi3-5 gl1 tt5-1* (X and dashed line). The germination assay was done with M_8 (for mutant 1, 2 and 3) and M_7 (for mutant 4) seeds and a 5-day cold treatment; each point represents three replicates of 30 to 80 seeds ± SE.

It has been reported that the original *lec1* mutants were occasionally viviparous (Meinke, 1992) which suggests the lack of primary dormancy. We observed some dormancy in the modifier lines since in several cases germination increased upon dry storage, before decreasing due to loss of viability. This was also observed in the progeny of the backcrosses of these lines with L*er* (data not shown).

Genetic analysis

To determine genetic segregation ratios the progeny of a mutant x non-mutant cross has to be analyzed. The distribution of the trait values studied over all individuals from such a cross might result in a conclusion about monogenic or polygenic inheritance of the trait. The phenotype of all, mutant and modifier, F_2 plants was based on the germination phenotype of their F_3 seeds, in *abi3-5* or *lec1-3* mutant background, at two different time points after harvest. For the evaluation of the data from both germination experiments a scatter diagram was made in which for each F_2 plant the percentages from both experiments were plotted against each other (Fig 4.6). One might then expect clouds of data points, in separate groups, by which the individuals carrying homozygous or heterozygous alleles could be clearly distinguished. Based on the segregation ratios obtained from the backcross of the selected genotypes to L*er*, no conclusions could be drawn about the mode of inheritance since no discrete classes could be observed.

To determine recessiveness/dominance and allelism, the *sua* mutants were crossed to *abi3-5 gl1 tt5-1* and among each other. The subsequent germination assays of F_1 seeds showed that all mutations were recessive (Fig 4.7A) and that they were not allelic (Fig 4.7B), thus representing mutations at four different loci called *sua1 – sua4*. The data for the F_1's *sua3* x *sua2* and *sua2* x *sua1* seemed not conclusive, however in subsequent mapping experiments (Fig 4.8) four different loci were identified. A possible explanation might be that in these double heterozygotes recessiveness is less well expressed compared to both single heterozygotes. The recessive nature of the mutations and the mapping experiments indicate that improved storability was most likely due to intergenic suppressors. For the *sua4* mutant the original strong *abi3-5* allele segregated in the progeny of the *sua4* x L*er* cross also indicating that this is an intergenic suppressor.

Comparing germination of the F_3 seeds derived from backcrosses of the selected *lec1-3*/*abi3-5* modifier lines with L*er*, no clear-cut segregation ratios could be observed. Figure 4.6B and 4.6C show a scatter diagram of the germination of F_3 *abi3-5* mutant seeds for respectively abi/Wa and *abi*/Col lines. For *abi*/Sei, *abi* /Eil and *abi*/Gu, comparable data were obtained (data not shown). The absence of discrete classes does not allow a conclusion about mono- or polygenic inheritance. The distribution for the *lec*/Sha2 F_3 seeds is shown in Figure 4.6D, only for the *lec1-3* mutant. Similar patterns were observed for *lec*/Sha2 and *lec*/Gu (data not shown).

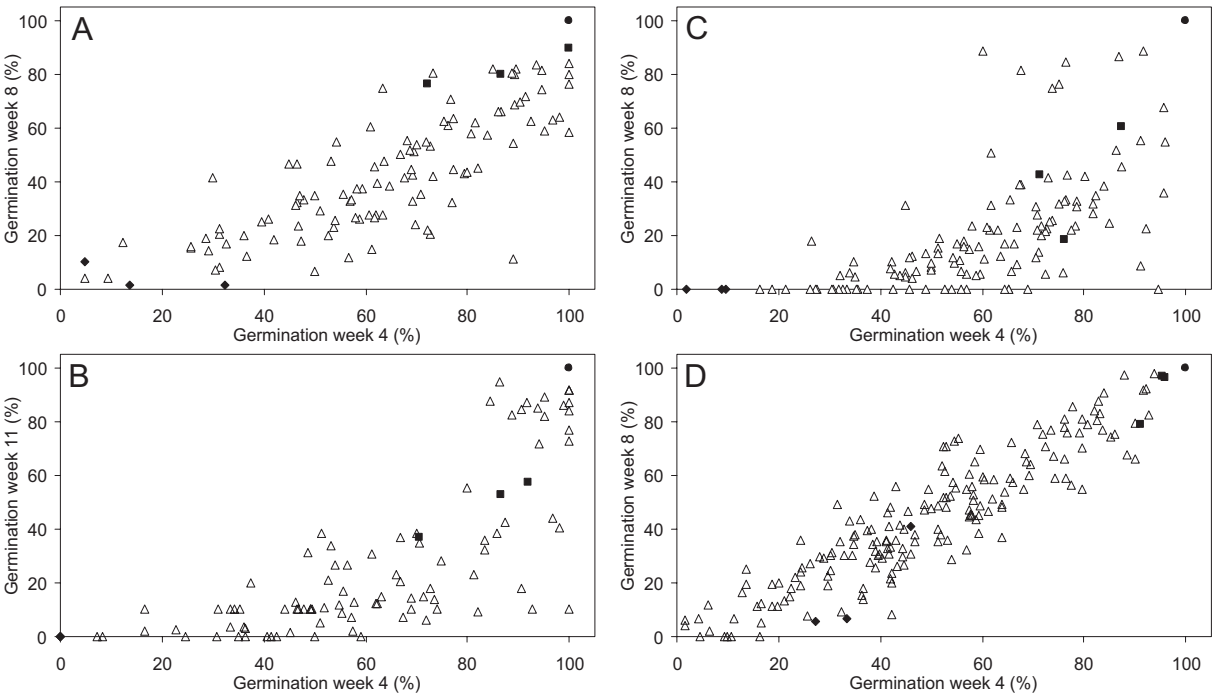

Figure 4.6. Scatter diagrams showing germination percentages, of mutant *abi3-5*/*lec1-3* F_3 seeds. Germination percentages were determined at two different time points after harvest, of the individuals of 4 mapping populations and respective controls. **A**: *sua1* crossed to L*er*: wild-type (L*er*; closed circle), *sua1* (closed squares), *abi3-5 gl1 tt5-1* (closed diamonds) and 104 individuals from the cross *sua1* x L*er* (open triangles). **B**: *abi*/Wa crossed to L*er*: wild-type (L*er*; closed circle), *abi*/Wa (closed squares), *abi3-5 gl1 tt5-1* (closed diamond) and 90 individuals from the cross *abi*/Wa x L*er* (open triangles). **C**: *abi*/Col crossed to L*er*: wild-type (L*er*; closed circle), *abi*/Col (closed squares), *abi3-5 gl1 tt5-1* (closed diamonds) and 120 individuals from the cross *abi*/Col x L*er* (open triangles). **D**: *lec*/Sha2 crossed to L*er*: wild-type (L*er*; closed circle), *lec*/Sha2 (closed squares), *lec1-3* (closed diamonds) and 170 individuals from the cross *lec*/Sha2 x L*er* (open triangles).

Mapping

Crude map-positions were determined using the bulked segregant analysis (Michelmore et al., 1991). DNA was isolated from F_2 plants with either the *abi3-5* or *lec1-3* phenotype. DNA from a mixture of six to eight best performing and six to eight worst performing F_2 plants was used to find linkage with specific markers. Individuals with an enhanced storability were chosen from the top right corner in the scatter diagram and poorly storable individuals from bottom left. When linkage was found, the individual F_2 plants were tested with markers from the region where linkage was detected.

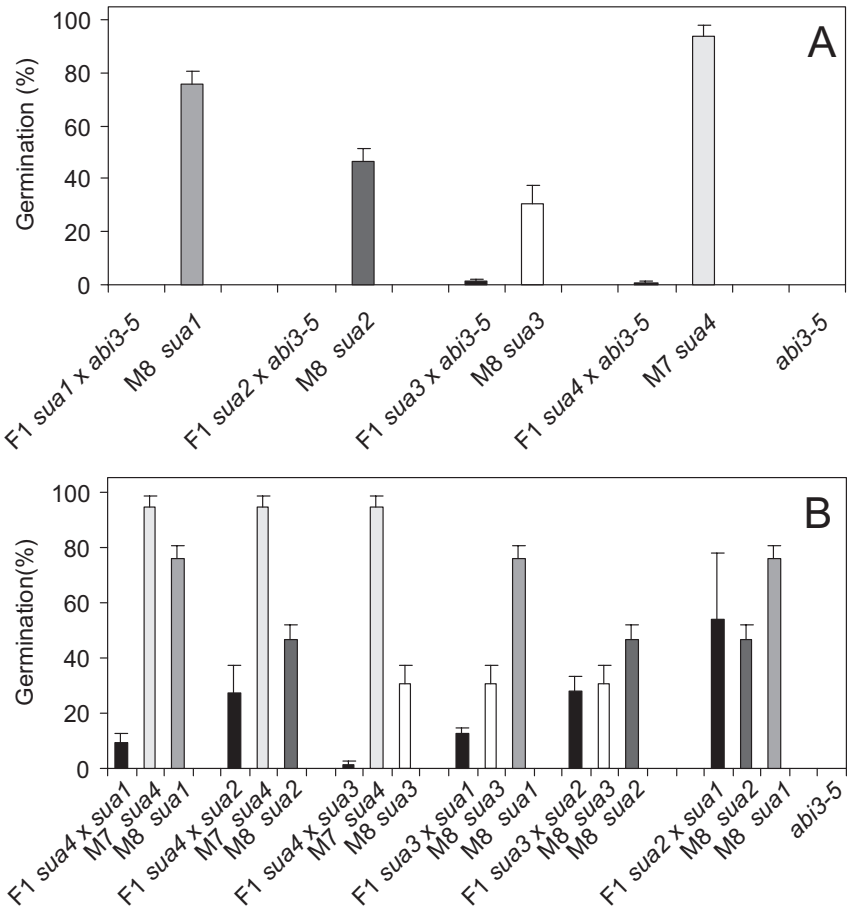

Figure 4.7: Germination percentages of F_1 (black bars) seeds that had been crossed to *abi3-5 gl1 tt5-1* (**A**) and among each other (**B**) and respective controls. In both A and B controls are represented by gray bars: *sua1*, dark gray: *sua2*, white bars: *sua3* and light gray: *sua4*. Assays were conducted after two months of dry storage, each bar represents 4 replicates of 10 to 40 seeds ± SE.

A mapping population for the sua mutants was made by crossing them to the Colombia *glabra* (Col-5). Analysis showed that *sua1* (Fig 4.8) is linked to marker MBK5 at the bottom of chromosome 5, *sua2* to marker mln, also on chromosome 5 and *sua4* showed linkage to nga112, near the bottom of chromosome 3. These linkages were confirmed by two to three other markers in the same region. In case of *sua3* only significant linkage could be shown to cHALFd; this was just above threshold. Confirmation using the markers msat5-22 and MBK could not be shown since the linkage with these markers was not statistically significant. In all cases the L*er* alleles were found to be associated with better storability, indicating that no modifiers from Col were mapped. This might have been expected, since a natural modifier was isolated from Col. However, the fact that no absolute linkage could be detected with any marker might be due to the segregation of such modifiers as well as due to the variable expression of the trait. Consequently some of the presumed homozygotes for storability might have been in fact heterozygote F_2 plants (F_3 seeds).

To map the modifier loci from the accessions all eight modifier-lines were backcrossed to L*er*, and analyzed using the bulked segregant analysis and confirmation by analysis of individual F_2 plants. This analysis was restricted to markers that were non-L*er* in the selected lines since the modifiers are, in contrast to the *sua* mutant, derived from the accessions.

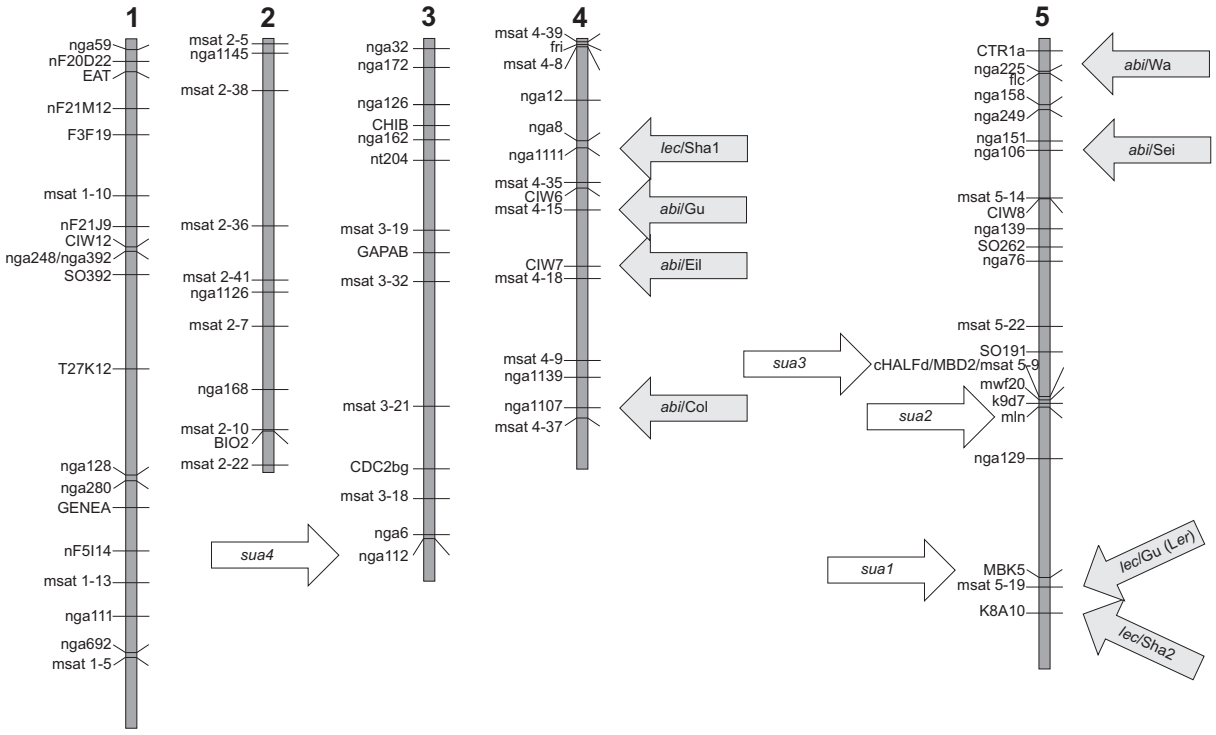

Figure 4.8. Map depicting the map-positions of the various mutant modifiers (white arrows on left side of the chromosome) and natural modifiers (gray arrows on right side of the chromosome). Arrows point towards the marker that showed the strongest significant linkage as determined with the Chi-square (see materials and methods). The map is based on the physical distances and the markers are placed according to the physical position of the BAC on which they are situated.

This resulted in a number of map-positions on chromosome 4 (Fig 4.8); for *lec*/Sha1 around marker nga1111, for *abi*/Gu around marker msat4-15, for *abi*/Eil around marker CIW7 and for *abi*/Col around marker nga1107. On chromosome 5 map-positions were found for *abi*/Wa, around nga225, for *abi*/Sei around nga106 and for *lec*/Sha2 around K8A10. Because the modifiers from the two Sha selections were located at two different regions they should represent different modifier loci. These map-positions were confirmed by checking individual samples again with one to three markers near the positions that had been found. No linkage of better storable lines with the Gu-0 allele could be found for the progeny of the *lec*/Gu x L*er* cross. However, a strongly significant linkage ($P < 0.05$) was found with L*er* alleles at the bottom of chromosome 5. This indicates a synergistic interaction between L*er* alleles at a locus at the bottom of chromosome 5 and Gu-0 alleles at one or more loci that have not yet been detected. In all other cases enhanced storability was, as expected, associated with alleles of the accession improving the phenotype of *lec1-3* and *abi3-5*, while the non-storable phenotype was linked to L*er* alleles.

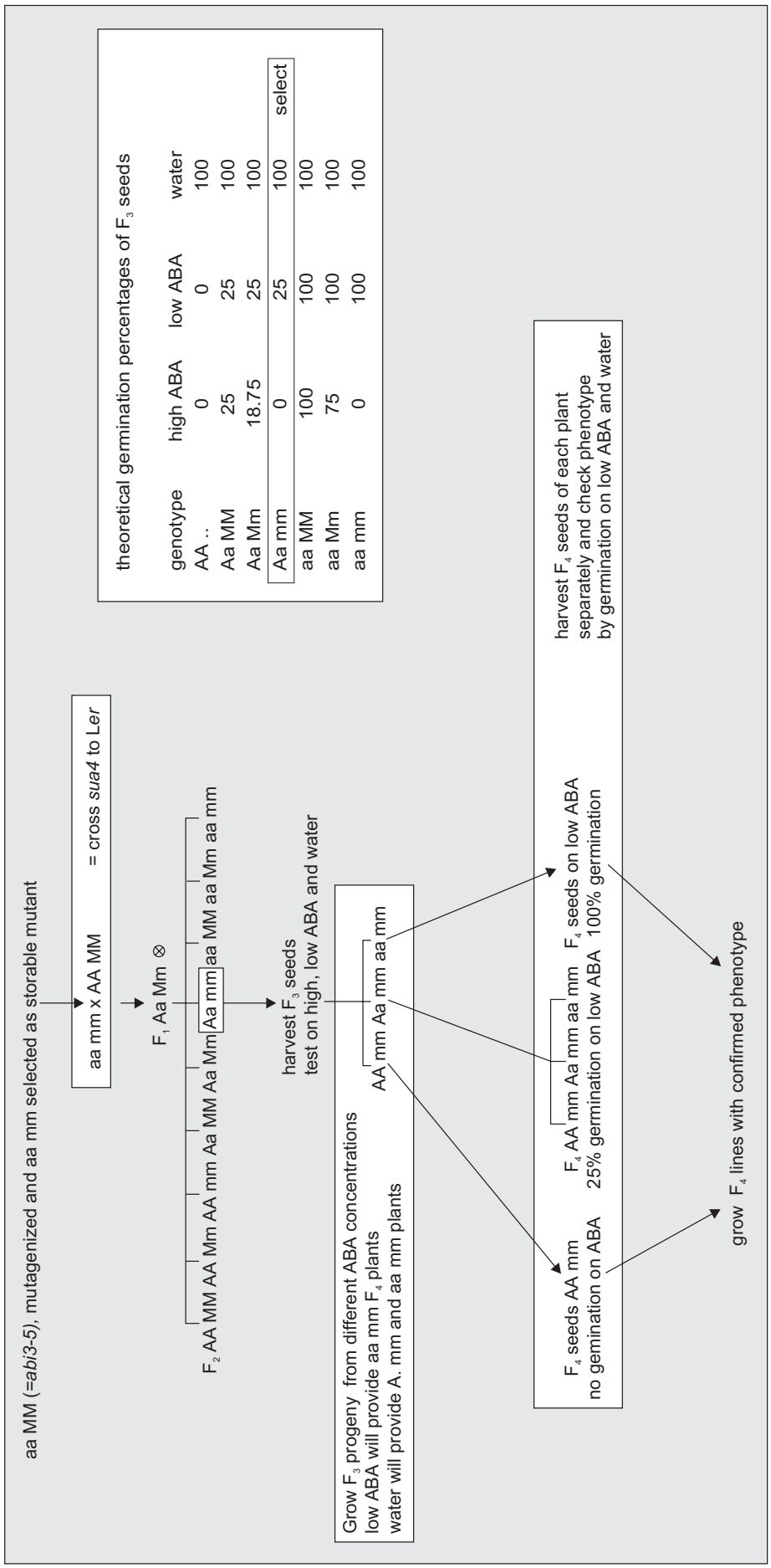

Figure 4.9. Procedure to select *sua4* without the *abi3-5* mutation. This selection is based on the aberrant germination phenotype of *sua4* on ABA, as depicted in the theoretical germination phenotype of F_3 seeds. Homozygous *abi3-5* is represented by aa, m represents the mutant alleles and M the wild-type alleles of the modifying mutation in *sua4*.

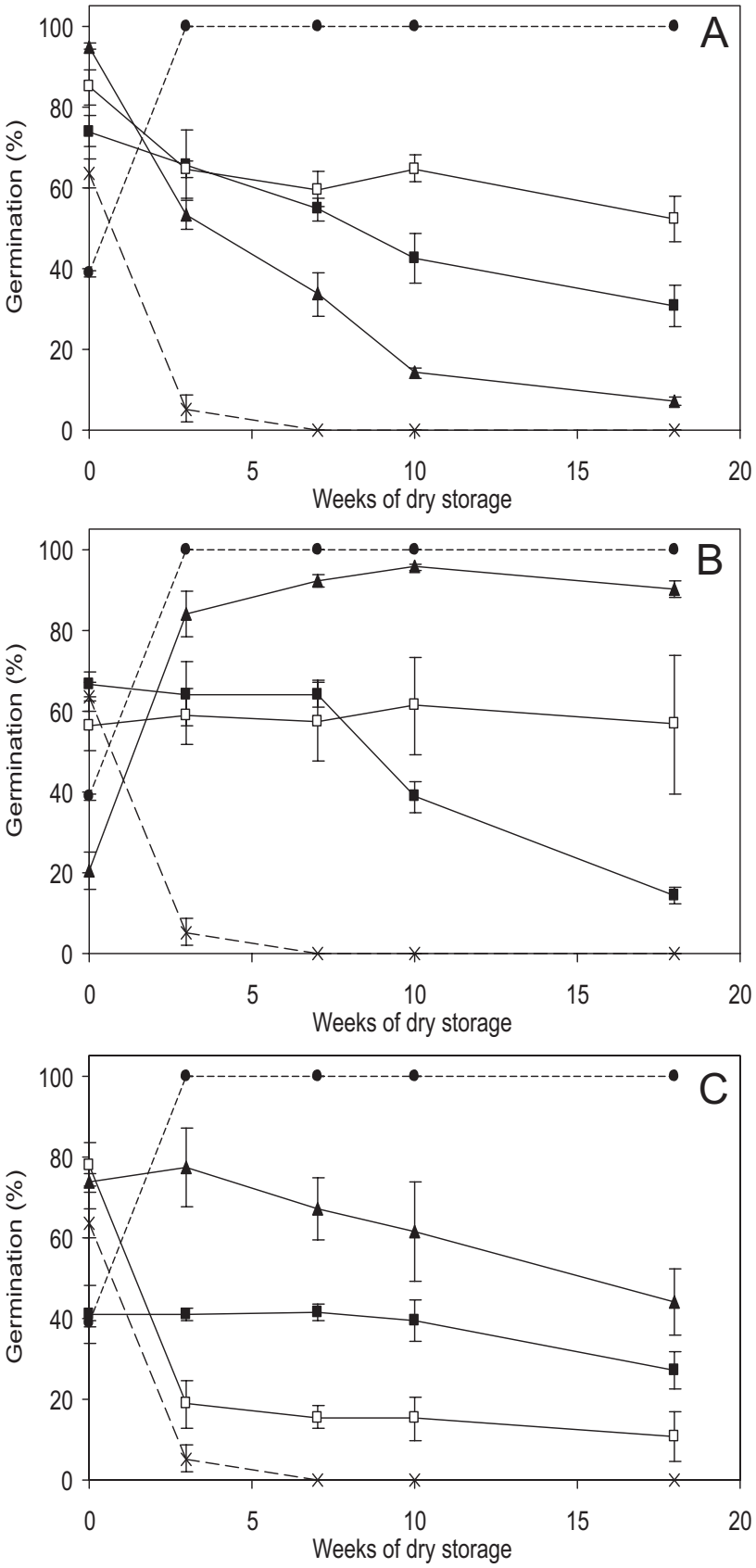

Figure 4.10. Germination behavior of two different reselected modifier line seeds harvested from mature dry siliques and after subsequent dry storage; data are averages of three replicates of 40 to 80 seeds ± SE. A three-day cold treatment was applied to break dormancy; still the control L*er* shows a release of dormancy phenotype. This might also be present in some of the reselected lines. **A**: F_6 generation of *lec*/Sha1 x L*er* 1 and 2 (closed and open squares) with the controls wild-type (L*er*; closed circles and dashed), *lec1-3* (X and dashed line), and F_{14} of *lec*/Sha1 (closed triangles). **B**: F_6 generation of *lec*/Sha2 x L*er* 1 and 2 (closed and open squares) with the controls wild-type (L*er*; closed circles and dashed), *lec1-3* (X and dashed line) and F_{14} of *lec*/Sha2 (closed triangles). **C**: F_6 generation of *lec*/Gu x L*er* 1 and 2 (closed and open squares) with the controls wild-type (L*er*; closed circles and dashed), *lec1-3* (X and dashed line) and F_{14} of *lec*/Gu (closed triangles).

Selection of backcrossed lines with and without *lec*/*abi*

All *sua* mutants and modifier selections were backcrossed to L*er*, to select sister-lines with and without the *lec1-3* or *abi3-5* mutations. The procedure followed is depicted in the pale gray part of Figure 4.3. Isolation of the *sua1, 2* and *3* mutant modifiers without *abi3-5* was performed similar to the natural modifier selection (Fig 4.3). The procedure for *sua4* was different since this mutant showed a different ABA response phenotype (Fig 4.5), which could be used for selection as depicted in Figure 4.9.

The selection procedure for sister-lines with and without the *lec1-3* or *abi3-5* could only be finalized for the *lec1-3* lines. Figure 4.10 (A, B and C) showed that after backcrossing and subsequent selection the storability phenotype in the *lec1*/modifier lines could be maintained. Two separate selections of *lec*/Sha1 x L*er*, *lec*/Sha2 x L*er* and *lec*/Gu x L*er* were obtained. For some of these lines the germinability first increased during storage, probably due to dormancy since also L*er* samples showed that a dormancy breaking treatment of 3 days at 4°C, was not sufficient to break dormancy of freshly harvested seeds. The selected *LEC1-3* modifier sister-lines were considered homozygous for the natural modifier based on the phenotype of their *lec1-3* mutant sister-lines.

Controlled deterioration test (CDT)

To test the effect on storability of the modifier in wild-type background *LEC*/modifier lines were subjected to a CDT. In a CDT seeds are exposed to a high humidity and temperature for a short time, this treatment mimics natural aging. The result of this CDT (Fig 4.11A) showed that Sha is more tolerant to high humidity and temperature for a prolonged period compared to L*er* and Gu-0. The backcrossed *LEC*/modifier lines showed that their survival rate is similar to L*er* except for *LEC*/Sha1x L*er* (1), which resembles the Sha wild-type. The backcrossed selections of *LEC*/Gu did not show an improvement of the survival rate in a CDT compared to the wild-type Gu-0. The results were confirmed by the percentage of abnormal seedlings as fraction of the total number of germinated seeds (Fig 4.11B).

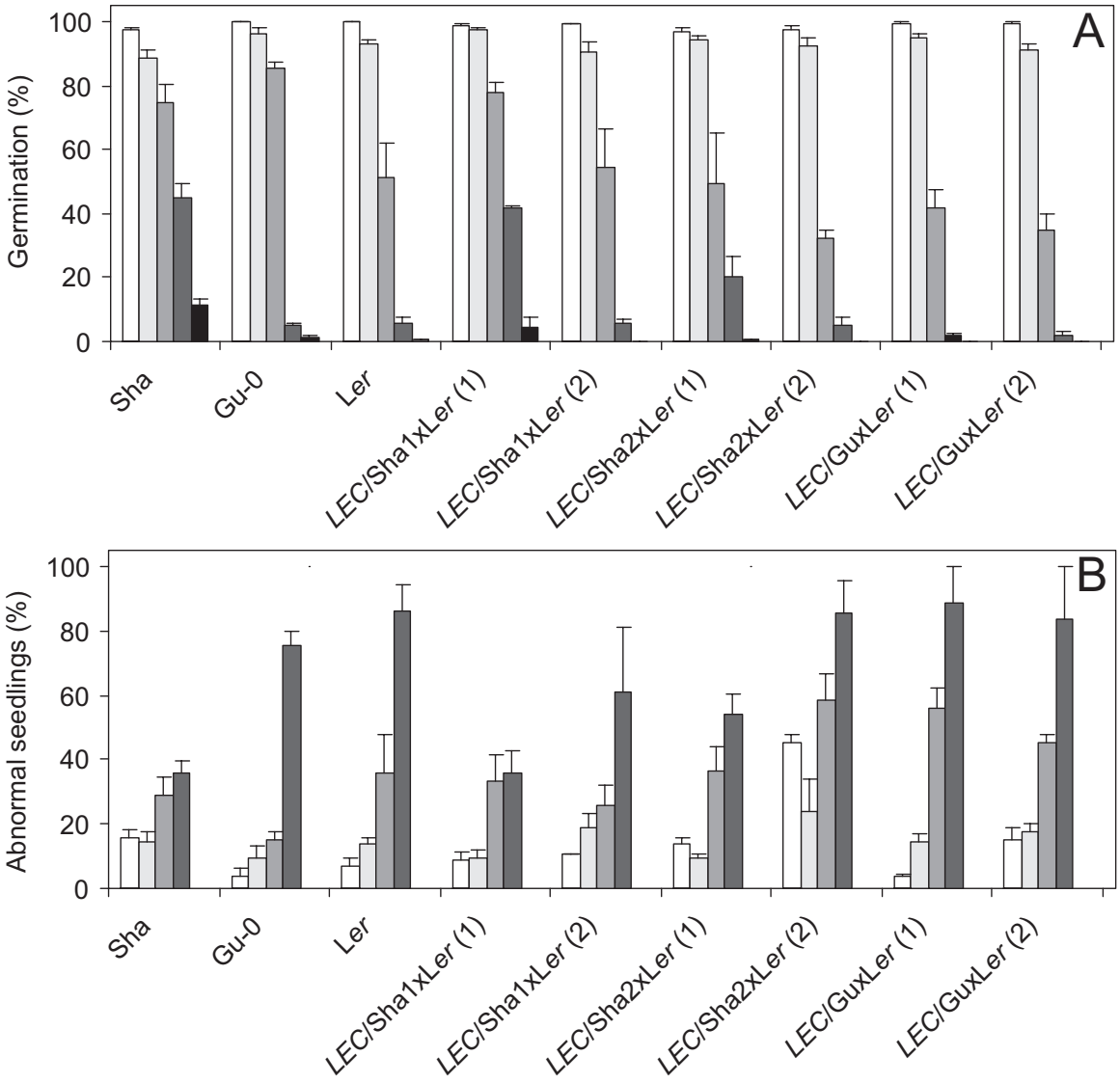

Figure 4.11. CDT performed with lines containing the modifier in *LEC1* background. Seeds were stored at 42°C and at 85 %RH for 0 (white), 1 (light gray), 3 (gray), 5 (dark gray) and 7 days (black); each bar represents three replicates of 100 seeds + SE. **A**: shows the germination percentage after 14 days. **B**: depicts the percentage of abnormal seedlings as percentage of total germination on each respective day; percentage abnormal seedlings at day 7 is not depicted.

Conductivity measurement

Damage during dry storage of seeds that may result in loss of viability has often been associated with damage to membranes which can be measured as leakage of electrolytes. We tested whether the enhanced storability of the *lec*/Sha1, *lec*/Sha2 and *lec*/Gu could be due to improved membrane stability. We correlated germination after 7 weeks of storage with conductivity after 8 weeks of storage (Fig 4.12). It was observed that the decrease in germination in the *lec1-3* mutant modifier lines, including *lec1-3*, is negatively correlated (r = -0.72) with conductivity. This indicates that the improved storability was, at least partially, due

to increased membrane stability since most of the *lec1-3* mutant natural modifier lines showed a lower leakage combined with a higher germination in comparison to the *lec1-3* mutant.

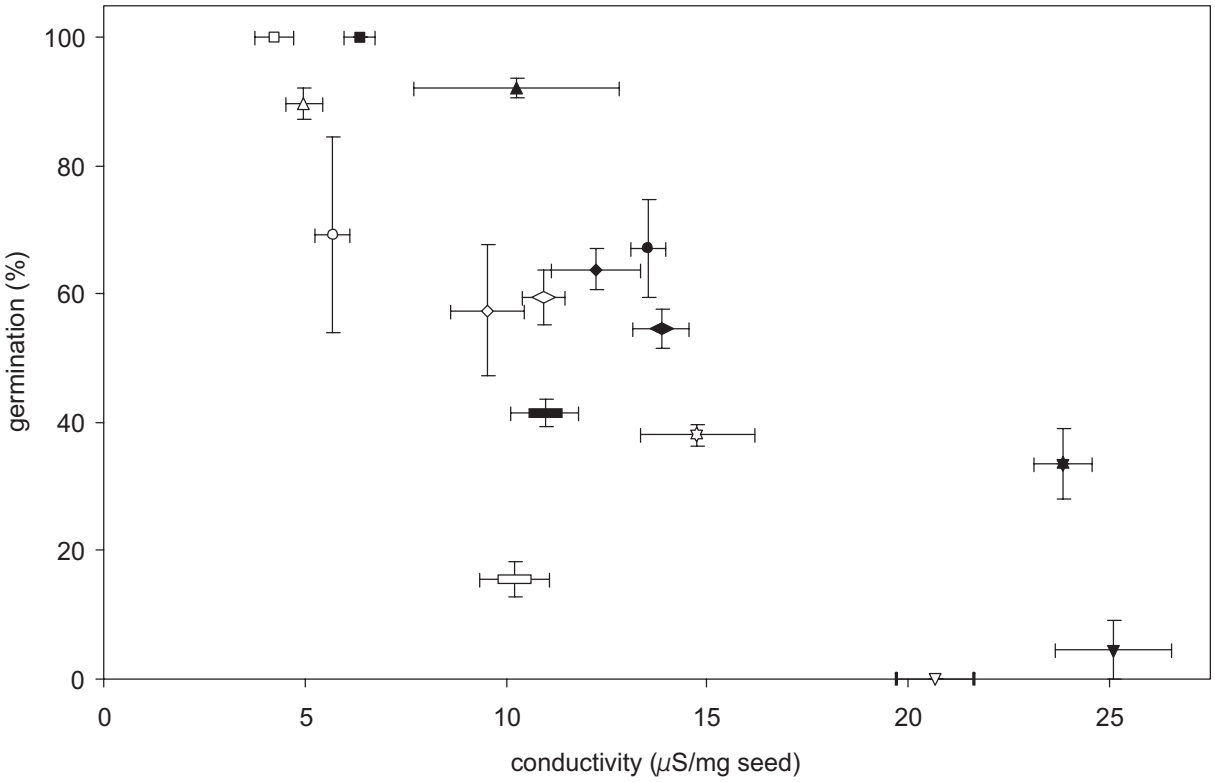

Figure 4.12. Correlation of germination after 7 weeks of storage, and conductivity after 8 weeks of dry storage. Open and closed symbols represent two independent measurements and in the case of the backcrossed lines two different isolations. Each measurement is based on 3 replicates of 40 to 80 seeds for germination and 5 mg of seeds for conductivity ± SE. Genotypes are represented by squares: L*er*, stars: *lec*/Sha1, upright triangles: *lec*/Sha2, circles: *lec*/Gu, downward triangles: *lec1-3*, oblong diamonds: *lec*/Sha1xL*er* (closed: 1, open: 2), diamonds: *lec*/Sha2xL*er* (closed: 1, open: 2), rectangles: *lec*/GuxL*er* (closed: 1, open: 2).

Discussion

Mutant selection in a “sensitized” background is described as selection for genetic modifiers of a certain process by isolating new mutants in the background of an existing mutant in the process under study (Matin and Nadeau, 2001) or in a different wild-type strain. This method will allow the identification of mutants, which in wild-type background show no or only a very subtle phenotype. Here we used the maturation defective *lec1-3* and *abi3-5* mutants to find genes involved in desiccation tolerance and seed longevity. The *abi3-5* mutant was used in both a mutagenesis and natural modifier selection. The *lec1-3* mutant was only used in the selection of natural modifiers.

Selection by mutagenesis of novel mutants that modify specific mutations is common in Arabidopsis research (Page and Grossniklaus, 2002) and has also been applied using the ABA insensitive *abi1* mutant acting as the “sensitized” selection background. The gibberellin insensitive, *sleepy1* (*sly1*) mutant was isolated as a suppressor of *abi1*, since the *sly1abi1* double mutant seeds do not germinate in the presence of ABA (Steber et al., 1998). Using again the *abi1* mutant Beaudoin et al. (2000) isolated an enhancer of the ABA response of which the seeds were able to germinate on a higher concentration of ABA as compared to the single *abi1* mutant seeds. In a similar way they isolated a suppressor of the ABA response of which the seeds were more sensitive to ABA at germination. This screen identified mutants of the ethylene-signaling pathway and indicated an interaction between ABA and ethylene signaling that was not known before. *Green seed*, an enhancer of *abi3-1* was isolated because of its green seed color in the sensitive *abi3-1* background. The single mutant did not show the green seed color, but did show a small effect on seed longevity (Clerkx et.al., 2003).

In Arabidopsis a few examples can be found where modifiers of specific mutations were found to be present in natural populations. The floral meristem identity mutant *cauliflower1* (*cal1*) was identified as an enhancer of *apetala1* (*ap1*), when the *ap1* allele was crossed into Wassilewkija (Ws) background. The *cal1* single mutant has no phenotype in Ws, because *AP1* compensates for its loss in Ws (Bowman et al., 1993; Kempin et al., 1995). Extreme late flowering in Arabidopsis accessions mostly depends on a dominant *FRIGIDA* (*FRI*) allele, the expression of which is depending on active alleles at the *FLOWERING LOCUS C* (*FLC*) locus (Koornneef et al., 1994). This implies that variation for *FRI* is only detectable in a sensitive *FLC* background and *FLC* only in a *FRI* background. The Cape Verde Islands (Cvi) allele of the blue light photoreceptor cryptochrome2 confers earliness in short days in a L*er* genetic background, whereas the Cvi accession itself still flowers late in this condition (Alonso-Blanco et al., 1998a; El-Assal et al., 2001), making L*er* the sensitive background for the detection of allelic variation at this locus. The study by both mutagenesis

and selection of modifiers from natural populations, in the sensitive *lec1-3* and *abi3-5* mutants, provided lines of which the seeds showed a prolonged longevity.

The many pleiotropic effects of the *abi3* mutants indicate that the ABI3 protein affects many processes during seed maturation and germination, including the storability of seeds. In this report we describe the isolation of both natural and mutational induced modifiers, affecting some aspects of the pleiotropic *abi3* mutant phenotype. The ABA sensitivity is affected strongly in the *sua4* mutant, which might indicate a modifier of the ABA response itself. However, in the other *sua* mutants ABA sensitivity is not affected in a major way. Other aspects of the *abi3-5* mutant phenotype such as the lack of seed dormancy are unaffected in all *sua* mutants. The largest effect that can be seen is the increased storability, which was the main selection criterion. However, how these modifiers will behave without the *abi3-5* mutation is not known.

The *lec1-3* mutants also show a large number of pleiotropic effects, such as the accumulation of anthocyanin in cotyledons, an activated shoot apical meristem, altered embryo shape, trichromes on the cotyledons, defects in seed storage reserve accumulation, and a reduced desiccation tolerance making the mutant seeds non-storable (Lotan et al., 1998; Harada, 2001). Our main interest concerns the genetic regulation of storability and acquiring desiccation tolerance since the latter is an important aspect of seed longevity (Wilson Jr, 1995; Ingram and Bartels, 1996). Identifying modifiers of the storability and reduced desiccation tolerant phenotype of the *lec1-3* seeds might provide insight in this process. The modifiers could either directly influence the target, acquiring storability and desiccation tolerance, or could function in the pathway leading towards storability. Characterization of the *lec1-3* mutant modifier lines has shown a decrease in electrolyte leakage upon imbibition. Increased leakage of electrolytes indicates a problem with cellular membrane integrity, which coincides with the finding that *lec1* seeds have a reduced macromolecular stability as shown by FTIR analysis (Wolkers et al., 1998). We therefore conclude that the increased storability could at least partially be due to increased membrane stability. Isolation of the modifiers in *LEC1* background and subsequently testing these in a CDT did not show an enhanced survival of these seeds compared to their L*er* wild-type. These observations might indicate that the modifier does not influence the target for acquiring longevity directly, but affects steps in the pathway leading towards desiccation tolerance and subsequent storability.

In case LEC1 acts as a suppressor of a negative regulator of desiccation tolerance, a reduced expression of the latter will only be observed in a *lec1* mutant background and not in wild-type. Furthermore, when desiccation tolerance has a different mechanistic basis than longevity and is only a prerequisite for the latter, one does not expect to see an effect on longevity when desiccation tolerance is normal as is in the wild-type. Only when desiccation

tolerance and longevity have mechanistic aspects in common one may expect to see an effect on longevity in the lines containing modifiers in a non-mutant background. Other scenarios explaining the lack of phenotype in the non-mutant modifier lines, such as the difference in the expression of paralogues are also possible. Gene redundancy originates from genome or gene duplications. Thereafter, this is followed by the independent evolution of the duplicated genes. This might have lead to a difference in their expression pattern both with respect to tissue and temporal specificity or to level of expression. Different accessions might also differ in expression pattern of these paralogues, in which case a duplicated gene from a genetic background where it is expressed to a higher level could act as a suppressor of a mutant phenotype for the other gene in another accession. One example of a *LEC1* paralogue is *L1L*, which shows 83% sequence similarity of the B-domain of *LEC1* at the protein level. In *lec1* mutants *L1L* is expressed indicating that endogenous *L1L* is not sufficient to completely substitute for the *LEC1* gene. However, ectopic expression of *L1L* under the 35S promoter suppresses aspects of the *lec1* mutant phenotype (Kwong et al. 2003). A higher expression of *L1L* in another Arabidopsis accession might then be able to suppress some of the pleiotropic effects, resulting in an enhanced storability. The modifier isolated from Shakdara, sha2 on chromosome 5, could be an allele of *LEC1-LIKE* (*L1L*) since this modifier is located in the vicinity of *L1L* (Kwong et al., 2003). Other candidates may be the recently isolated mutants: *chotto1* (*cho1*) and *cho2* (Nambara et al., 2002). These genes are necessary for the correct ABA responsiveness in seeds and *cho1* was mapped to the bottom of chromosome 5 to a position, which is similar to the position of *sua3*.

Our mapping experiment shows that there is a relatively small number of positions where the modifiers, of storability, are located. These co-locations might suggest that the same genes are involved. The limited number of loci also, possibly, indicate that there is a small number of genes with relative large effect that contribute to the effects observed. Another indication for the limited number of genes might be that the region near nga1111, where the Sha1 (top chromosome 5) modifier improves storability, was also identified in a QTL mapping experiment using L*er*/Sha recombinant inbred lines. In this population the survival rate in a CDT was enhanced by a Sha allele at this locus (Clerkx et al. Chapter 5). It cannot be excluded that more genes segregate that have remained undetected. This is suggested by the differences between the storability of the original lec/modifier line and lines reselected from a cross of these lines with L*er* (Fig 4.10). The relatively small mapping populations and the variable and quantitative expression of the trait in general will not allow detection of genes for which allelic variation has only small effects. The variable expression of the trait and the presence of more undetected modifiers also make the map positions relatively inaccurate.

Further characterization, like more accurate mapping and also further physiological and molecular analysis such as the analysis of the expression of genes and proteins known to be affected by both mutants might help in defining the processes affected by these genes.

Acknowledgements

We would like to thank Dr. Folkert Hoekstra for the use of the conductivity meter and for critically reading the manuscript.

Materials and methods

Plant material and selection

The *lec1-3* mutant of *Arabidopsis thaliana* was isolated from an En/I transposon containing L*er* population (Aarts et al., 1995; Raz et al., 2001) and contains an insertion 124 bp upstream of the start codon. The line used is a stable mutant line from which the transposase source is absent. The *abi3-5* allele has been described by Ooms et al. (1993) and Bies-Ethève et al. (1999) and was combined with the morphological markers *gl1* and *tt5-1*, which are like *abi3*, also located on chromosome 3. The accessions used for the selection of modifiers were C24 (CS906), Columbia (Col, CS907), Cape Verde Islands (Cvi, N8580), Drahonin (Dra, N1116), Dijon (Di-G, CS910), Eilenburg (Eil-0, CS6693), Enkheim (En-2, CS1138), Eringsboda (Eri-1, CS22548), Gudow (Gd, N1184), Gluckingen (Gu-0, N1212), Koln (Kl-5, N1284), Landsberg *erecta* (L*er*, NW20), Niederzenz (Nd-1, CS1636), Nossen (No-0, CS1394), Rschew (RLD-1, CS913), Slavice (Sav-0, N1514), Seis am Schlern (Sei-0, N1504), Shakdara (Sha, CS929), Warschau (Wa-0, CS6885), Wassilewkija (Ws-1, CS2223). Within the siliques of F_1 plants a segregation of mutant and non-mutant F_2 seeds could be observed for both *abi3-5* and *lec1-3* just before the seeds color turns brown. Mutant seeds can be distinguished in the case of *abi3-5* by their green color and ABA insensitivity and in the case of *lec1-3* by their anthocyanin accumulation. Seeds homozygous for *lec1-3* were removed from the siliques, which are sterilized by dipping them in 70% (V/V) ethanol, and germinated on half strength Murashige and Skoog medium supplemented with 1% (W/V) sucrose. Homozygous *abi3-5* seeds were selected by sowing F_2 seeds on filter-paper soaked with 10 µM ABA. Approximately 30 homozygous F_2 plants were grown for each cross. F_3 seeds were harvested from all individual F_2 plants and selection was performed on the basis of germination of mutant seeds after 1 month of dry storage (ambient RH, probably not above 40%, and temperature). F_3 plants were grown from lines whose seeds showed a clearly higher germination percentage than seeds from control *lec1-3* or *abi3-5 gl1 tt5-1* plants. The F_4 progeny of 3 plants per selected F_3 line was re-tested allowing a more stringent selection. The F_3 lines, which clearly showed improved seed storability were also backcrossed to L*er* to select the isolated modifiers without the *lec1-3* and *abi3-5 gl1 tt5-1* mutations and to perform a mapping. The various selection and genetic analysis procedures are summarized in Figure 4.3 and 4.9.

Mutagenesis

To perform the first mutagenesis approximately 12000 (240 mg) *abi3-5 gl1 tt5-1* seeds were allowed to imbibe for a few hours in water agar (0.2% w/v). Half of the imbibed seeds were then exposed to 200 Gy, the other half to 300 Gy of gamma-radiation. The M_1 seeds were then sown in batches of approximately 100 seeds per pot. M_2 seeds were harvested as bulks per pot and were harvested, together with non-radiated controls. These controls were stored (ambient conditions) and sown at regular intervals; when all control seeds were dead (natural aging) all batches of M_2 seeds were sown on Petri-dished containing 5 µM ABA, to prevent any wild-type contaminants from germinating. All

seeds that had germinated were transferred to soil and M_3 seeds were harvested. These M_3 seeds were again stored until non-radiated controls were dead; germinating M_3 seeds were transferred to soil. M_4 seeds were harvested, and their storability was tested and compared to that of *abi3-5 gl1 tt5-1* seeds, thereafter the best lines were selected. Mutants selected from different M_2 batches were considered to have arisen from independent mutation events. The 300 Gy dose appeared too high because no mutants were isolated from the seed batch that had been exposed to this dose. A second mutagenesis treatment was performed with approximately 9000 seeds (180mg) and these seeds were exposed to the lower dose of 150 Gy, thereafter the same selection procedure was followed.

Culture conditions and seed germination assays

Seeds were sown in Petri-dishes on water-saturated filter paper and incubated in a growth chamber at 25 ° C. After 2 days of incubation, germinated seeds were transferred to soil and cultivated in an air-conditioned greenhouse (18°- 23°C, 16 h photoperiod). Growing *lec1-3* and *abi3-5* plants on half strength MS before transfer to the greenhouse, allowed a much better survival of the seedlings compared to seeds germinated on filter-paper. Seeds sown on half strength MS medium were sterilized using the vapor-phase seed sterilization protocol in a desiccator jar, placed in the fume-hood. Seed were placed in opened Eppendorf tubes in a desiccator jar, then a 250 ml beaker containing 100 ml bleach was placed inside and 3 ml concentrated HCl added to the bleach. Then the desiccator jar was closed and the seeds sterilized by the chlorine gas. After 2 to 3 hours the jar was opened and the Eppendorf tubes were closed until use (http://plantpath.wisc.edu/~afb/vapster.html). Seeds were harvested from mature fully desiccated siliques. Thereafter seeds were stored at ambient conditions until use. All germination assays were done in 3 replicates of 50-80 seeds. If necessary, imbibed seeds were stored for up to 7 days at 4°C and subsequently transferred to a growth chamber (25°C, 16 h light period). Germination was scored 7 d after the start of incubation at 25°C. To determine the sensitivity of germination to ABA 40-80 seeds were harvested from siliques of approximately the same age, all 3 replicates were sown on filter paper soaked with a range of ABA concentrations in 6 cm plastic Petri-dishes. The imbibed seeds were stored for 5 to 6 days at 4°C and subsequently incubated in a growth chamber (25°C, 16 h light period). Germination was scored 7 d after the start of incubation at 25°C.

To determine whether the *sua* mutants were dominant or recessive they were crossed to wild-type (L*er*). To test their allelism they were crossed among each other. F_1 seeds were harvested from mature ripe siliques and stored under ambient conditions. Germination percentage of 4 replicates of 10 to 40 seeds were determined after two months of storage; the data for these 4 replicates were thereafter averaged.

Controlled deterioration tests

Seeds that had been harvested from mature ripe siliques were used in a CDT. The CDT (Tesnier et al., 2002) was performed as follows: seeds are equilibrated at 85% relative humidity (15°C), 0 day

controls are immediately dried back at 32% relative humidity. Treatment is done by storing the seeds (at 85% RH) for a number of days at 40°C (here 1, 2, 3 and 5 days), after which these seeds are dried back at 32% RH (20°C) and stored at circa 4°C until germination was tested. Three replicates of 100 seeds, from two different F_6 wild-type *LEC*/acc x L*er* per modifier line, were germinated on water-saturated, thick filter paper placed on a "Copenhagen" germination table at 25°C and with an 8-hour light period per day. To improve detection of radical protrusion a black filter membrane (Schleicher and Schuell, Dassel, Germany, ref.-nr: 409712) was placed between the seeds and the filter paper. The seeds were covered with a transparent bell-shaped cover to prevent drying and the final germination was evaluated 14 days after transferring the seeds to the "Copenhagen" germination table.

Conductivity measurement

Seeds from mature dry siliques were harvested; 5 mg were weighed from two different backcrossed F_6 *lec1-3* mutant modifier lines. They were placed at 100% RH overnight to reduce possible imbibitional damage. Thereafter, conductivity of all seed samples was measured in a CM100 conductivity meter (Reid & associates, Durban, South Africa). Germination was determined the same seed lots at approximately the same time after storage as the conductivity was measured.

DNA isolation

DNA was isolated from greenhouse grown plants, one plant per line per plot. The Bernatzky and Tanksley (1986) protocol was adapted for rapid extraction of small quantities. Flower buds were harvested in liquid nitrogen and grinded in 330µl of a preheated (65°C) extraction solution (125µl extraction buffer (0.35 M Sorbitol. 100 mM Tris, 5 mM EDTA, pH 7.5 (HCl) together with 175 µl Lysis buffer (200 mM Tris, 50 mM EDTA, 2 M NaCl, 2 % (w/v) cetyl-trimethyl-ammonium bromide) to which 30 µl Sarkosyl (10% w/v)) was added. The mixture of crude plant material and extraction solution was incubated for 30 minutes at 65°C with occasional shaking. Hereafter, a solution of 400µl chloroform/isoamyl alcohol (24:1) was added and vortexed. After centrifuging for 5 minutes at maximum speed in an Eppendorf centrifuge the water phase was transferred to a new tube. An equal amount of isopropanol was added to the tube and to precipitate the DNA the tube was inverted carefully several times. After 10 min centrifugation at maximum speed in Eppendorf centrifuge the water-alcohol mixture was discarded and the pellet washed with 70% cold ethanol. The pellet was left to dry and dissolved in water containing RNAse A and incubated 30 minutes at 37°C; thereafter it was stored in the refrigerator. Later stages required more DNA , which was isolated from media grown seedlings. This DNA was isolated using the Wizard ® magnetic 96 (Promega; #FF3760) DNA isolation kit.

Mapping and PCR analysis

To locate the various mutations and modifiers in the case of the mutants they were crossed to Colombia *glabra* (Col-5, CS1644) and for the modifiers a backcross was made to L*er*. All markers depicted on the map were checked for polymorphisms among the various accessions used. Mapping was done according the bulked segregant method described by Michelmore et al. (1991). We found that 25 cycles improved the possibility to see differential amplification. DNA was bulked of 5 to 8 F_2 plants that were selected on the basis of a high germination of their progeny after 4 and 8 weeks of storage. Also DNA was bulked from 5 to 8 F_2 with low germinating progeny. In the analysis a heterozygous sample of F_1 plants, was taken along, as were the parental controls. The first quick check was done with 3 markers randomly distributed on each chromosome, in total 15, with the bulked segregant method. When putative linkage was observed, all individual samples, ranging from 7 to 15 high and 5 to 15 low germinating samples, were amplified with the same marker and a Chi-square test performed to confirm the linkage found; next to that more markers, 1 to 3, in the same area were tested to confirm the linkage.

All PCR protocols were, if necessary, adapted for a specific primer combination or else a standard protocol of 35 cycles 94°C 30 sec, 50°C 30 sec and 72°C 30 sec.
Markers were taken from the Tair database (http://www.arabidopsis.org) or taken from (http://www.inra.fr/qtlat/msat). Some PCR markers were not found in one of these databases, these are specified in Table 4.1. The FLC marker was provided by Tony Gendall (John Innes Centre , Norwich , UK; Gazzani et al., 2003). Novel markers were developed on the basis of the Col and L*er* sequence in Arabidopsis public databases (Jander et al., 2002) see Table 4.1. Mutant and wild-type *abi3-5* alleles could be amplified with the following dCAPS primer pair, 5'-GAATCTCCACCGTCATGGTCAC and 5'-ACAAGAACCTCCTCTGTCTCGC, thereafter wild-type alleles can be distinguished by digestion with HphI. Three primers were used to identify *lec1-3* mutants, 5'-CTTGCCTTTTTTCTTGTAGTG, 5'-GACATACAACACTTTTCCTTAAAG, and 5'-GTCCCAAGTTTTAGTGTGAGG. These were used as one mix containing all three primers, the first two amplify a ± 0.9 kb product only in *lec1-3* since the first primer is specific to the En/I transposon. The last two will amplify a product only in L*er* of ± 1.3 kb, potentially these primers could amplify a product in *lec1*-3 as well, although this was never observed. Using all three primers in one reaction also allows the identification of heterozygous plants.

Table 4.1: New markers used in mapping mutants and modifiers

chr	marker	BAC	primer 1 (5' to 3')	primer 2 (5' to 3')	Polymorphisms	Origin
1	F3F19	F3F19	CGTCCAGACACTGACATTGGTTTTAGG	CCACTCACGTTCAGTGGGGTTAAACT	Col, Sei, Eil, Wa, Gu, Sha	Genetics
4	FRI	F6N23	CATGTCGTAATCATGCAACC	GAAGATCATCGAATTGGC	Col, Sei, Eil, Wa, Gu, Sha	Tony Gendall
5	FLC	T31P16	CATTGGATAACTAATCTTTGAGC	CAGGCTGGAGAGATGACAAAA	Wa, Sei, Sha	Tony Gendall
5	MBD2	MBD2	TCACCGAACCTCTGCTTTCTC	AAAGTAGGACCGGCGACGAG	Col, Eil	Genetics
5	mwf20	MBD2B	ATTTGGGCCATGTAACTAATGC	AAAGTCAATGCAACCGTACAAC	Col, Sei, Eil	Genetics
5	mln	MLN1	GGCTGTTGCTCTCGTCCTTG	CCAGTCACCAGAACCAGCTTG	Col, Wa, Gu	Genetics
5	K9D7	K9D7	GCTGTTGTAATTTGTGATAGGG	CATATGCCACGTTTCTTAATAG	Col, Sei, Eil, Wa, Sha	Genetics
5	K8A10	K8A10	AATGCCAAGGATCAAAAGTGTT	GATGATCGGAGGAAAATGAAAA	Sha	Genetics

Genetics: marker was developed at the laboratory of genetics
Polymorphism accession with different product length from L*er* are mentioned (some might not have been tested with all accessions used)
Marker provided by Tony Gendal (Gazzani et. al., 2003)

Chapter 5

Analysis of natural allelic variation of Arabidopsis seed quality traits between the accessions Landsberg *erecta* and Shakdara, using a new recombinant inbred line population

Emile J.M. Clerkx, Mohamed E. El-Lithy, Elizabeth Vierling, Gerda J. Ruys, Hetty Blankestijn-De Vries, Steven P.C. Groot, Dick Vreugdenhil, Maarten Koornneef

Abstract

Quantitative trait loci (QTL) mapping was used to identify loci controlling various aspects of seed quality during storage and germination. Similar locations for QTLs controlling different traits might be an indication for a common genetic control of such traits. For this analysis we used a new recombinant inbred line (RIL) population derived from a cross between the accessions Landsberg *erecta* (L*er*) and Shakdara (Sha). A set of 114 F9 RILs was genotyped with 65 PCR based markers and the phenotypic marker *erecta*. The traits analyzed were: dormancy, speed of germination, seed sugar content, seed germination after a controlled deterioration test (CDT), hydrogenperoxide (H_2O_2) treatment and on abscisic acid (ABA). Furthermore the effects of heat stress, salt (NaCl) stress, osmotic (mannitol) stress and natural aging were analyzed. For all traits one or more QTLs were identified, with some QTLs for different traits co-locating. The relevance of co-location for mechanisms underlying the various traits is discussed.

This chapter has been submitted for publication

Introduction

Seeds of good quality are undamaged and have a high rate of germination, high viability and will produce vigorous seedlings under various environmental conditions. Physiological damage results in poor seed quality. The nature of this damage is variable, e.g. short-term deterioration in the field is different from long-term deterioration during storage, which in turn is different from mechanical damage (McDonald, 1999). All organs, that make-up the seed, are susceptible to physiological damage: the seed coat, which is of maternal origin and acts as a physical and chemical barrier, the embryo, the endosperm and the cotyledons. Seed quality has been related to various seed properties such as color, weight and membrane composition. The correlation between seed quality and these traits is often species or in some cases even variety specific (Dickson, 1980; McDonald, 1999). The ability of seeds to withstand stresses that occur while stored is one aspect of seed quality. To some extent these stresses may resemble those that occur when imbibed seeds are exposed to unfavorable conditions during germination. Seed quality may also be related to the level of deterioration of the seeds during storage, moisture content and temperature being the major environmental factors influencing this trait.

Very little is known about the genetic basis of differences in seed quality because this trait is affected by environmental effects during seed formation, harvest and storage, and is probably controlled by several genes. Only recently some studies on this complex trait have been initiated using QTL mapping in crop plants like cabbage (*Brassica*; Bettey et al., 2000), tomato (*Lycopersicon sp.*; Foolad et al., 1999), rice (*Oryza sativa*; Cui et al., 2002; Miura et al., 2002), barley (*Hordeum vulgare*; Mano and Takeda, 1997) and *Sorghum bicolor* (Natoli et al., 2002).

Arabidopsis can be a good model species for the identification of genes controlling seed quality, since it is amenable to both classical and molecular genetic studies (Meinke et al., 1998; Page and Grossniklaus, 2002). This, combined with the multitude of publicly available molecular tools, including a complete genome sequence, implies that the cloning of genes can proceed relatively quickly in Arabidopsis.

To investigate the genetics of seed viability and vigor one can identify mutants that either have improved or reduced seed quality. Among Arabidopsis mutants that have a poor seed quality, evidenced by their rapid loss of viability upon storage, are mutants that affect seed maturation such as *leafy cotyledon1* (*lec1*)*, lec2, fusca3* (*fus3*) (Holdsworth et al., 1999; Finkelstein et al., 2002) and strong *abscisic acid insensitive3* (*abi3*) alleles (Ooms et al., 1993). Mutants with smaller effects are weak alleles of *abi3* (Koornneef et al., 1984; Bies-Ethève et al., 1999), the *aberrant testa shape* (*ats*) mutant (Léon-Kloosterziel et al., 1994), the *transparent testa* (*tt*) mutants (Debeaujon et al., 2000) and the *green seed* mutant (Clerkx

et al., 2003). Other sources of genetic variation can be found in naturally occurring populations. Arabidopsis is widely distributed in the world, thus encountering substantial variation in growth environments. Therefore, phenotypic variation is expected to reflect the genetic variation important for adaptation to specific environments as summarized by Alonso-Blanco and Koornneef (2000). Seed viability and vigor are of complex nature and thus an interesting trait to be studied using a QTL approach. The advent of efficient molecular marker technologies and specific statistical methods in the past decade have allowed that map positions of QTLs can be established with sufficient accuracy (Alonso-Blanco and Koornneef, 2000). Mapping QTLs requires a segregating population for which a genetic map has been established and an accurate phenotyping of the trait. Immortal mapping populations such as RILs are very useful because each genotype can be tested repeatedly and by applying different test systems. The latter allows studying the pleiotropic effects of loci, which are suggested by co-location of QTLs for different traits.

A test commonly used to assess seedling viability and vigor is a controlled deterioration test (CDT), in which seeds are stored at high relative humidity and temperature. For Arabidopsis this test is described by Tesnier et al. (2002). Several mutants, but also different accessions, show different responses to the treatment indicating the presence of genetic variation for the response to this test. Bentsink et al. (2000) confirmed this by identifying several QTLs for survival after a CDT using a L*er*/Cape Verde Islands (Cvi) RIL population. Quesada et al. (2002) simulated the effect of drought stress on germination by using NaCl and identified QTLs controlling this trait in the L*er*/Colombia (Col) RIL population.

In dry desiccation tolerant seeds, protection of proteins and membranes during desiccation occurs by water replacement. Water molecules are replaced by sugars at the hydrogen bonding sites to preserve the native structure of proteins and the spacing between phospholipids (Hoekstra et al., 2001). Besides sugars, heat-inducible hydrophilic late embryogenic abundant (LEA) proteins might also play a role in protein and membrane stabilization by acting as molecular chaperones (Hoekstra et al., 2001). Bettey and Finch-Savage (1998) investigated the role of two subclasses of LEAs, dehydrins and small heat shock proteins (sHSP) in *Brassica* seeds. Dehydrins did not show a positive relationship with seed performance. However, the protein HSP17.6 showed a positive correlation with seed performance (Bettey and Finch-Savage, 1998). Further evidence for role of sHSP's could come from the fact that in Arabidopsis cytosolic sHSP's appear to respond to specific developmental signals associated with the acquisition of desiccation tolerance (Wehmeyer and Vierling, 2000)

Seeds normally germinate in a wide range of temperatures. It seems that the major determinant of germination is the availability of water (Bewley and Black, 1994). Under stress conditions such as extreme temperatures, salt stress and water deficit, germination is

delayed or completely inhibited depending on the stress intensity and the genetic background (Foolad et al., 1999). To differentiate between loci involved specifically in germination under stress, Foolad et al. (1999) and Bettey et al. (2000) determined QTLs for speed of germination and argued that these loci are important for germination in general and are not specifically affected by stress.

In the present study we analyzed the genetic control of the response to various stress treatments applied during seed storage and imbibition to investigate if tolerance to such factors has a common genetic basis. This study was performed using a newly developed RIL population derived from a cross of L*er* x Sha. L*er* originates from Poland and Sha from the Shakdara mountain range in Tadjikistan at 3400 m elevation (Khurmatov, 1982). Preliminary experiments indicated that Sha is one of the accessions most tolerant to various seed stresses. We were able to identify QTLs for various seed stresses viz., germination after a CDT, heat treatment and germination on NaCl, mannitol, H_2O_2 and ABA. Furthermore QTLs were identified for seed-dormancy, seed sugar content, natural aging and germination speed. Some of these QTLs co-locate which might indicate possible common genetic regulations.

Results

The L*er*/Sha RIL population

Using 114 F9 lines a genetic map (Fig 5.1) was generated using 66 markers distributed over the genome, which has a total length of 378 cM. There was an average distance between markers of 5.7 cM and there were 14 segments having a genetic distance between 10 and 14 cM (Table 5.1). Of all possible marker data points 1.2 % were not available, usually due to missing amplification or uncertainty in scoring. The average residual heterozygosity per locus was 0.7%, which was slightly higher than the expected 0.4% for an F9 generation. On average, L*er* alleles represented 54% of the alleles, certain regions showed a significantly distorted segregation ratio. These included the top of chromosome 1 (between nga59 and F3F19), chromosome 2 (between msat2-5 and msat2-38; M is msat in map, Fig 5.1), chromosome 3 (around markers CHIB and nt204), chromosome 4 (between marker C6L9-78 and CIW7) and chromosome 5 (around marker K8A10). Except for the region on chromosome 3, where Sha alleles were in excess, the bias was always in favor of L*er* alleles.

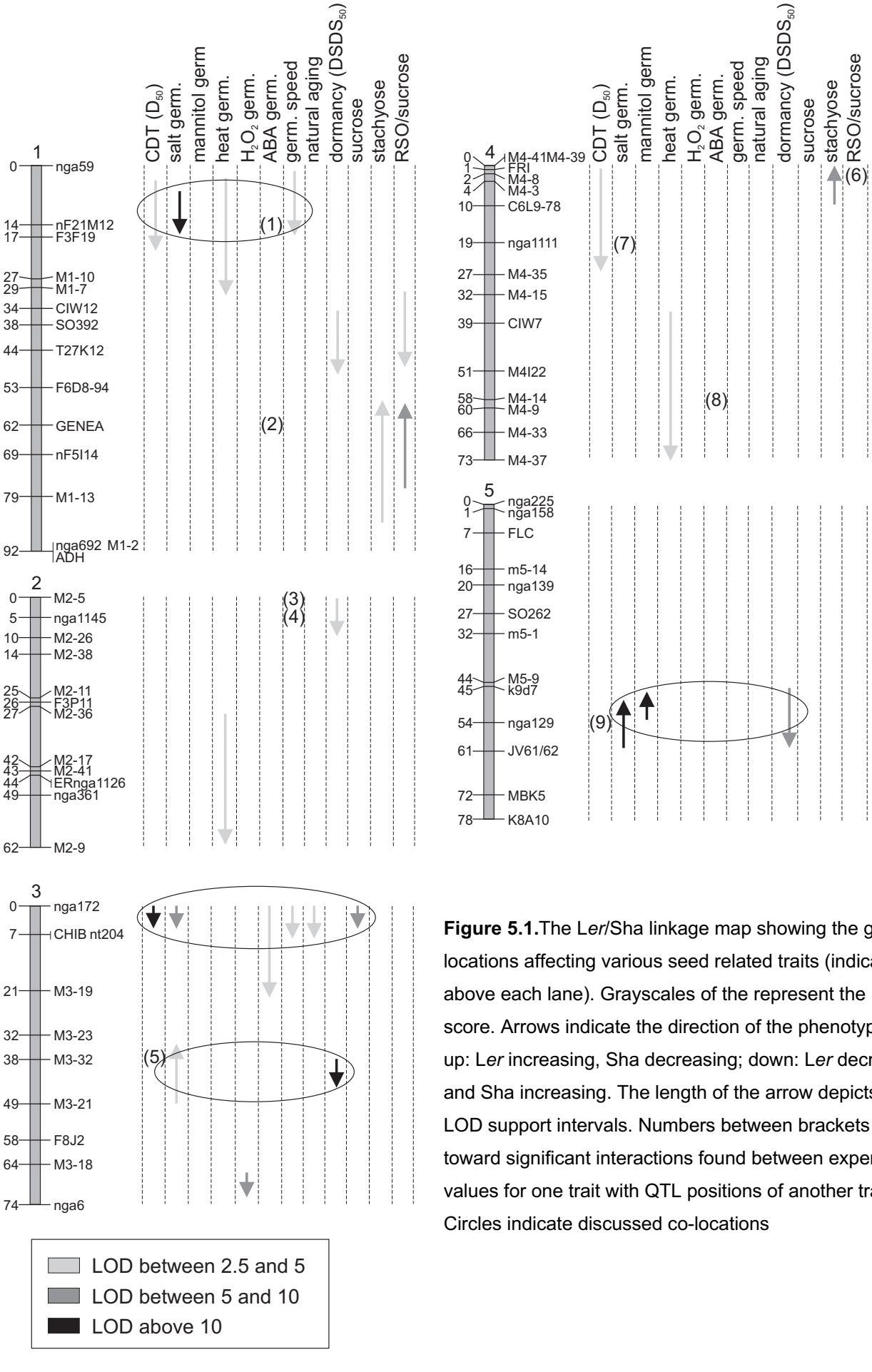

Figure 5.1. The L*er*/Sha linkage map showing the genetic locations affecting various seed related traits (indicated above each lane). Grayscales of the represent the LOD-score. Arrows indicate the direction of the phenotypic effect; up: L*er* increasing, Sha decreasing; down: L*er* decreasing and Sha increasing. The length of the arrow depicts the 2-LOD support intervals. Numbers between brackets point toward significant interactions found between experimental values for one trait with QTL positions of another trait. Circles indicate discussed co-locations

Table 5.1: Map features per chromosome and entire genome

chrom	total (cM)	kb/cM	avr. dist. /marker (cM)	gaps > 10 cM
1	92.3	308	6.2	4
2	61.7	293	4.7	3
3	74.1	311	7.4	4
4	72.6	239	4.8	1
5	77.7	339	6.0	2
total	378.5	299	5.7	14

Map distances were calculated with the JoinMap program
Physical distances were calculated using the center of the BAC in which the primer is situated

QTL mapping

For all traits analyzed in germination experiments broad sense heritabilities were estimated as the proportion of variance explained by between-line differences. These heritabilities ranged from 0.60 for germination on ABA up to 0.88 for seed dormancy (Table 5.2). Table 5.2 also shows the parental values and values obtained from F_2 seeds harvested from the two reciprocal F_1 hybrids indicating some reciprocal differences and suggesting some maternal effects for instance for dormancy (expressed as days of dry seed storage to reach 50% germination: $DSDS_{50}$: Alonso-Blanco et al. 2003). Frequency distributions of the various traits analyzed and the parental values are shown in the histograms in Figure 5.2. It was also clear from these histograms that some traits were not normally distributed. These were: D_{50} (the number of days CDT required to reach 50% germination), salt-, mannitol-, heat treatment-, H_2O_2-, ABA-, germination (respectively Fig 5.2 A, B, C, D, E and F) and speed of germination and germination after natural aging (respectively Fig 5.2 G and H). Improved normality was obtained by log-transformation of the D_{50} data. A probit transformation, to improve normality, was performed on the germination data for speed of germination, salt, mannitol, heat treatment, H_2O_2, ABA and natural aging. All transformations improved normality (data not shown) and the subsequent QTL analysis was performed with the transformed data.

Table 5.2: Trait values for L*er*, Sha and reciprocal F2 of L*er* x Sha and Sha x L*er*, along with the range of trait values for individual RILs and calculated heritabilities.

trait	L*er*	Sha	F2 (L*er* x Sha)	F2 (Sha x L*er)*	range	heritability
CDT (D_{50}; days)	1.3±0.1	2.8±0.2	1.3±0.1	1.6±0.0	0.5-5.1	0.82
Salt germ. (%)	8.7±1.2	21.2±0.4	13.7±2.1	12.8±6.3	0-79	0.82
Mannitol germ. (%)	97±1.6	79±6.6	83±3.4	82±8.2	10-100	0.79
Heat germ (%)	12±2.4	94±2.1	82±4.6	nd	1.3-100	0.93
H_2O_2 germ (%)	4.9±2.1	24±17	9.7±7.7	16±6.4	0-74	0.78
ABA germ (%)	57±18	97±0.8	82±17	93±2.4	59-100	0.60
Germ speed (%)	67±3.7	89±1.3	73±5.4	77±4.9	48-97	0.68
Natural aging (%)	89±2.0	96±1.4	86±8.2	89±3.9	64-100	0.69
Dormancy ($DSDS_{50}$; days)	9.6±1.0	31.8±7.4	19.0±3.5	29.4±4.0	3.4-80.5	0.88
Sucrose (µg/mg)	19.7±1.1	20.2±2.1	22.9±0.5	19.0±1.1	12.3-28.7	nd
Stachyose (µg/mg)	0.6±0.1	0.4±0.3	0.2±0.0	0.5±0.1	0-1.3	nd
RSO/suc.	0.06±0.01	0.06±0.02	0.03±0.00	0.05±0.01	0.01-0.11	nd

nd: not determined

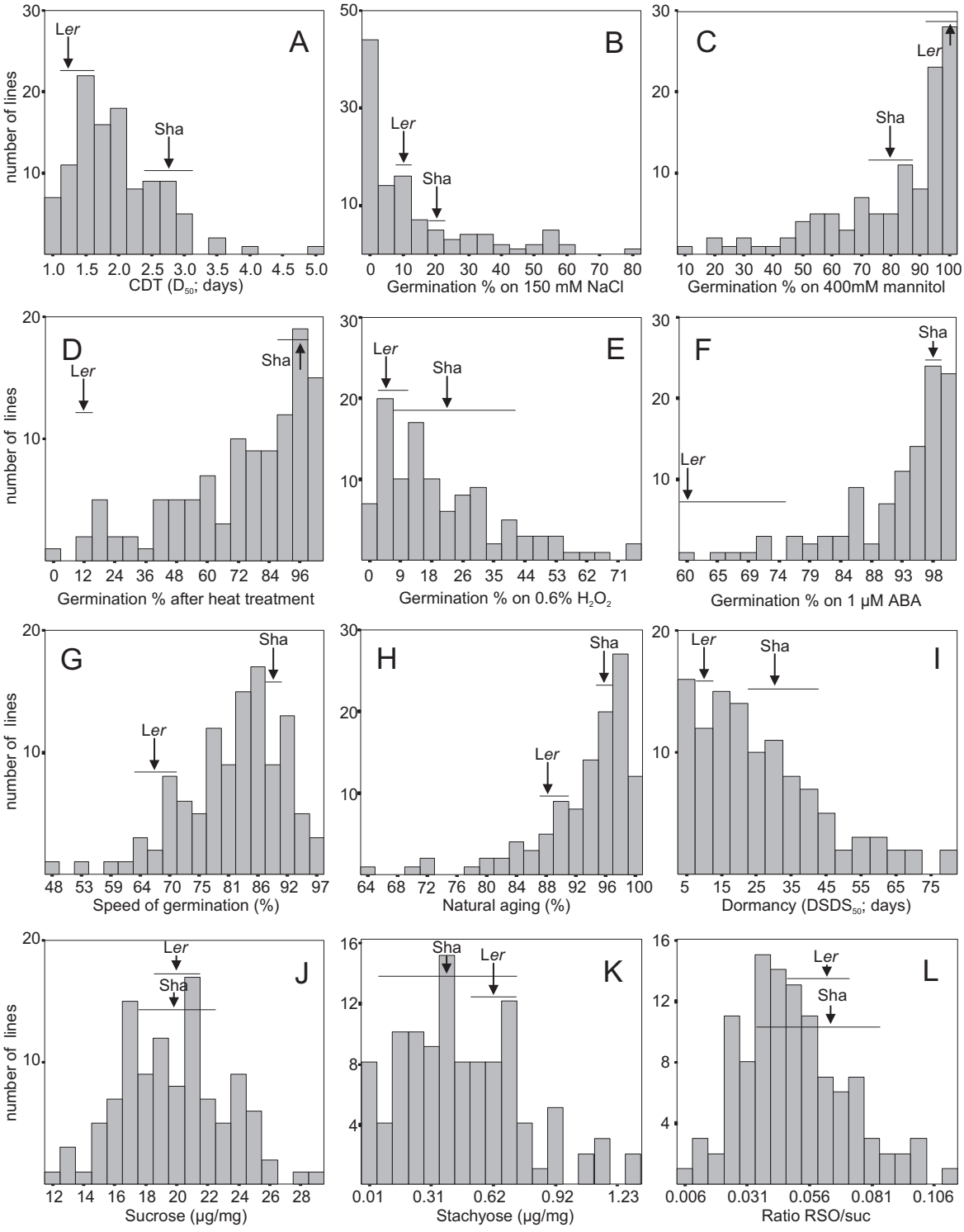

Figure 5.2. Frequency distributions of non-normalized data of all traits in the L*er*/Sha RIL population. Calculated number of days CD-treatment to reach 50% germination: D_{50} (A). Germination percentages on 150mM NaCl (B), 400mM mannitol (C), after an 8-hour 50°C treatment (D), on 0.6% H_2O_2 (E), and on 1 µM ABA (F). The speed of germination (G) and natural aging as the germination percentage after 20 months of storage (H). The calculated number of days of dry seed storage needed to reach 50% germination: $DSDS_{50}$ (I). Measurements of sucrose (J), stachyose (K) quantities and RSO to sucrose ratio (L). The average parental value is indicated with an arrow for both parents and the horizontal bar represents the SE for these parental values.

Mapping QTLs for non-stressed germination

To distinguish between loci specific for regulation of germination under stress vs. non-stress conditions the latter was determined using the germination speed under optimal conditions (Fig 5.2G). QTL analysis revealed two loci, viz., one on chromosome 1 and one on chromosome 3 that influenced germination speed (Fig 5.1). In total these two loci explained 28.6 % of the variance, and in both cases Sha alleles conferred a faster germination (Table 5.3).

Mapping QTLs for seed storability and natural aging

It was shown by Bentsink et al. (2000) and Tesnier et al. (2002) that a CDT can be used to identify genetic differences for seed storability. QTL analysis was performed using the D_{50} value. L*er* was more sensitive (lower storability) to the CDT conditions than Sha and most values for the RILs were in between the parental values (Fig 5.2A). Three QTLs were identified (Fig 5.1) affecting viability after controlled deterioration viz., on chromosome 1, 3 and 4 respectively (Table 5.3 for details). In total these loci explained 48.9 % of the variance. In all cases the Sha allele increased the number of days of treatment that the seeds could endure, which was in agreement with the limited transgression observed in the population (Fig 5.2A).

To test whether CDT mimics natural aging, seeds that had been stored for 20 months at ambient conditions were germinated (Fig 5.2H). The QTL mapping revealed only one QTL viz., on chromosome 3 (Fig 5.1), which explained 17.4% of the total variance (Table 5.3). The Sha allele conferred to a higher tolerance to prolonged storage, similar to the CDT QTL found at the same position.

Mapping QTLs for germination on NaCl, mannitol, H_2O_2 and after heat treatment

The ability of seeds to cope with water stress can be tested by germination on media containing NaCl (Fig 5.2B) or mannitol (Fig 5.2C). For germination on NaCl four QTLs, one on chromosome 1, two on 3 and one on 5 (Fig 5.1), were identified, in total they explained 59.4 % of the variance (Table 5.3). For two of these QTLs (chromosome 3 and 5) the L*er* alleles increased the germination percentage and for the other two the Sha alleles (chromosome 1 and 3) showed higher germination, which could explain the observed transgression (Fig 5.2 B). Germination on NaCl might also be explained as toxicity to sodium rather than the ability to cope with water stress. To distinguish between these two effects germination on mannitol was tested. Only one QTL for this trait was detected viz., on

chromosome 5 (Fig 5.1), which explained 49.4% of the variation (Table 5.3). The Sha allele decreased the germination percentage.

A factor commonly suggested in seed deterioration is reactive oxygen species (ROS). To mimic the effect of ROS we applied H_2O_2 during imbibition and subsequent germination (Fig 5.2E). QTL analysis revealed only one QTL viz., on chromosome 3, which explained 29.4 % of the variance (Table 5.3), the Sha allele increasing the tolerance to H_2O_2.

Imbibed Sha seeds showed a higher tolerance when exposed to high temperatures than L*er* seeds. Therefore, imbibed seeds of all RILs were exposed to 50°C for 8 hours and germination was scored after 7 days. Germination frequencies are shown in Figure 5.2D. Three QTLs were identified for this trait, viz., on chromosome 1, 2 and 4 (Fig 5.1), which explained 30.1% of the total variance observed. In all cases the Sha alleles confered a higher tolerance to the treatment (Table 5.3).

Mapping QTLs controlling dormancy and tolerance to ABA

Dormancy QTLs were mapped using the $DSDS_{50}$ value (Alonso-Blanco et al. 2003) for which in total 54.9% of the variance could be explained by 4 QTLs located on chromosome 1, 2, 3 and 5 respectively (Fig 5.1 and Table 5.3). In all cases the Sha alleles increased seed dormancy. The frequency distribution (Figure 5.2I) showed that there was some transgression beyond the Sha parental value, indicating that loci with minor effect, where L*er* alleles increase dormancy, might have remained undetected.

To test the sensitivity of the RIL lines to ABA we applied 1 µM ABA during germination (Fig 5.2F). This revealed only one QTL, viz., on chromosome 3 (Fig 5.1), which explained 11.2 % of the variation. The Sha allele conferred a higher tolerance to applied ABA (Table 5.3).

Mapping QTLs controlling seed soluble oligosaccharide content

QTL mapping was performed on the quantity of the three major soluble sugars in Arabidopsis seeds: sucrose and the raffinose series oligosaccharides (RSO: raffinose and stachyose) and the ratio of RSO to sucrose (Fig 5.2 J, K and L). Raffinose levels were low (data not shown) and no QTL for the content of this oligosaccharide could be detected. In four genomic regions QTLs were detected for sucrose and stachyose content. For sucrose a major QTL explaining 25.3% of the variation, was found on the top of chromosome 3 (Fig 5.1 and Table 5.3), the Sha allele increasing the sucrose content.

Table 5.3: Characteristics of QTLs detected in L*er*/Sha RIL population

Trait	chromosome	QTL position[a]	LOD	Percentage variance explained	Confidence Interval (cM)[b]	QTL effect[c]
						days
CDT (D_{50})	1	nF21M12	3.9	8.3	3-20	-0.41
	3	nga172	12.5	35.4	0-5	-0.81
	4	nga1111	4.3	10.1	1-26	-0.46
			total	48.9		
						% germ
Salt germ	1	nF21M12	12.1	27.4	6-16	-17
	3	nga172	5.2	10.1	0-5	-10
	3	msat3-32	2.8	5.1	34-49	7
	5	nga129	10.5	23.1	48-60	17
			total	58.6		
						% germ
Mannitol germ	5	k9D7	16.8	49.4	46-53	28
			total	49.4		
						% germ
Heat germ	1	nF21M12	3.7	11.5	3-31	-15
	2	msat2-41	2.6	7.9	28-60	-14
	4	msat4-14	2.9	8.8	36-73	-15
			total	30.1		
						% germ
H_2O_2 germ	3	nga6	8.7	29.4	69-74	-18.7
			total	29.4		
						% germ
ABA germ	3	nga172	2.8	11.2	0-23	-6.0
			total	11.2		
						% germ
Germ speed	1	nF21M12	4.3	13.9	0.5-17	-7
	3	nga172	5.0	16.5	0-6	-7
			total	28.6		
						% germ
Natural aging	3	nga172	4.6	17.4	0-6	-6
			total	17.4		
						days
Dormancy ($DSDS_{50}$)	1	T27K12	4	8	35-50	-11
	2	msat2-5	4	8.1	0-10	-10
	3	msat3-32	14.4	36.4	39-46	-21
	5	nga129	6.3	13.4	45-60	-14
			total	54.9		
						µg/mg
Sucrose	3	nga172	6.9	25.3	0-4	-3.44
			total	25.3		
						µg/mg
Stachyose	1	GENEA	3.9	11.7	56-85	0.21
	4	FRI	6.6	20.9	0-10	0.28
interaction[d]		GENEA x FRI		4.8		
			total	35.1		
						ratio
RSO/suc	1	CIW12	3	9.8	30-48	-0.013
	1	GENEA	6.6	22.9	57-77	0.02
interaction[d]		CIW12 x GENEA		3.3		
			total	28.1		

[a] co-factor nearest to highest LOD score: also co-factor fixed
[b] Positions given correspond to 2-LOD confidence intervals
[c] QTL effect of untransformed data
[d] interaction between QTLs calculated as indicated in "Materials and Methods"

Two QTLs, for which L*er* alleles increased the stachyose levels, were detected on chromosome 1 and 4 respectively (Fig 5.1 and Table 5.3). Interaction between these two loci was detected, explaining 4.8% of the variation observed. Sha alleles at the FRI marker had a synergistic effect when L*er* alleles at the GENEA marker were present. For the ratio RSO to sucrose two QTL with opposite allelic effects were detected, one of these apparently due to the higher stachyose content at the GENEA marker on chromosome 1 (Fig 5.1). The RSO to sucrose QTL at the position of marker CIW12 might have been influenced by a minor QTL for stachyose at the same position (LOD 2.2, data not shown). The interaction between the two loci, CIW12 and GENEA, explained 3.3% of the variance found for this trait. L*er* alleles at the GENEA marker had a synergistic effect when Sha alleles at the CIW12 marker were present (Table 5.3).

Other significant interactions

We hypothesized that similar physiological processes, like germination under different stresses, might have a common genetic basis. However, QTL mapping might not be able to detect a significant QTL due to statistical inaccuracy resulting from a low heritability and/or a relatively small population size. The latter effect is enforced by epistasis, a situation where variation for one locus is only observed in the background of a specific allele at another locus, thereby reducing the effective population size. Interactions between loci could be present and significant, despite the fact that the effects of the individual loci were not found to be significant. Therefore, we tested interactions at those positions where at least one of the traits showed a significant QTL using ANOVA. The CDT results were tested with the QTL positions of NaCl germination and showed a significant interaction between loci at the markers nga172 and at msat3-32 (number 5 in Fig 5.1) and also between loci at the markers nF21M12 and at nga129 (number 9 in Fig 5.1). The results of the germination on NaCl showed an interaction among loci at the CDT QTL positions at the markers nF21M12 and nga1111 (number 7 in Fig 5.1). Another interaction was found for the trait values of RSO to sucrose ratio and the QTL positions of stachyose between loci at the markers GENEA and FRI (number 6 in Fig 5.1). The ABA results were tested with QTL positions of salt tolerance and showed a significant interaction between loci at the markers nF21M12 and nga129 (number 1 in Fig 5.1); using the QTL positions of stachyose revealed an interaction between loci at the markers GENEA and FRI (number 2 in Fig 5.1). While using the QTL positions for heat germination revealed an interaction among loci at the markers nF21M12 and msat4-14 (number 8 in Fig 5.1). The speed of germination results showed two significant interactions with the QTL positions for dormancy, one between loci at the markers msat2-5 and msat3-32 and one among the loci at the markers msat2-5 and nga129 (respectively 3 and 4 in Fig 5.1).

Discussion

The L*er*/Sha RIL population

A linkage map has been generated for a novel Arabidopsis L*er*/Sha RIL population. These two accessions were used since preliminary tests (data not shown) had indicated that the accession Sha was very resistant to applied stress during germination. The map was made with 65 microsatellite, CAPS and indel markers (the latter being based on the Cereon database (Jander et al, 2002)), and the *erecta* mutation. The possibility of genotyping a population using only micro-satellites was already shown by Loudet et al. (2002) and allows perfect anchoring to the physical map of Arabidopsis. The order of the markers in the L*er*/Sha RIL population was in agreement with the published Col sequence. The relative length of the various chromosomes is similar to what has been published for maps based on other Arabidopsis RIL populations (Lister and Dean, 1993; Alonso-Blanco et al., 1998b; Loudet et al., 2002). The average distance per marker in kb per cM showed that only chromosome 4 was very different from the mean relative ratio of 320 kb per cM (Table 1), which was also seen in the Bayreuth (Bay-0)/Sha map described by Loudet et al. (2002). Large segregation distortion could compromise the QTL analysis. However, since the segregation ratios that we observed never exceeded a 1 to 1.8 ratio and we did not expect that this affected the present analysis. Similar biases in the segregation ratios were observed in other populations like L*er*/Col, L*er*/Cvi, Col/Kashmir and Bay-0/Sha (Lister and Dean, 1993; Alonso-Blanco et al., 1998b; Wilson et al., 2001; Loudet et al., 2002). The regions where distortion occurred were different from those regions where distortion was found in other populations.

Comparison of QTL positions with QTLs in previously analyzed Arabidopsis populations.

The finding of QTLs at similar positions for the same traits in different mapping populations gives an indication that allelic variation in different accessions might result from similar loci. However, the relative inaccuracy of the map positions obtained never excludes that in different crosses, distinct but closely linked genes segregate. Comparing the QTLs for storability found in the L*er*/Sha population to those found in the L*er*/Cvi population (Bentsink et al., 2000) revealed that the QTLs on chromosome 1 and 2 could be in the same regions. In both cases the L*er* alleles decreased the storability while Sha and Cvi increased the storability.

Comparing the germination rates found on medium supplemented with NaCl indicates that the L*er*/Sha RILs were much more sensitive to salt stress than the L*er*/Col lines which were tested at a higher salt concentration (Quesada et al., 2002). Therefore, it cannot be excluded that the difference in experimental parameters measures salt tolerance differently. Comparison of the QTLs found in both populations showed that two QTLs could be in the same region. In both populations the L*er* allele decreased germination for the QTL found on chromosome1. In the region where Quesada et al. (2002) located a QTL for days needed to reach 50% germination on salt-supplemented medium (QT_{50}), a QTL was also found in the L*er*/Sha RILs, although these had opposite effects. In the L*er*/Sha population, L*er* increases the tolerance and in the L*er*/Col, Col increases the tolerance. Thus, the relative effects of the alleles on salt tolerance could be: Col > L*er* > Sha, if this is due to the same locus.

Despite the low level of explained variance observed for seed sugar content, a comparison of the present data with those published by Bentsink et al. (2000) revealed that the QTL for stachyose on the lower arm of chromosome 1 in the L*er*/Sha population and in the L*er*/Cvi (Bentsink et al., 2000) population could be at a similar position. For sucrose content only one QTL could be detected, on top of chromosome 3, which was also detected in the L*er*/Cvi population (Bentsink et al., 2000).

The four dormancy QTLs detected in the L*er*/Sha RIL population co-located with QTLs previously identified in the L*er*/Col RIL population (van der Schaar et al., 1997) and all except one (chromosome 2) were also found in the L*er*/Cvi population (Alonso-Blanco et al., 2003). The absence of additional loci in this population might suggest that seed dormancy in Arabidopsis is controlled by a limited number of loci. Comparison of the quantitative effects of the individual QTLs between the L*er*/Sha and L*er*/Cvi populations, suggests that the QTL on chromosome 1 has a similar effect in both populations (Sha and Cvi increase the $DSDS_{50}$ with 11 and 12 days respectively). The same is found for chromosome 5 where the L*er*/Sha QTL should be compared to DOG7 in L*er*/Cvi (Alonso-Blanco et al., 2003). The Sha allele increases the $DSDS_{50}$ value with 14 days and Cvi by 15 days. However, for the QTL at chromosome 3 the increase in $DSDS_{50}$ of the Sha allele is much larger (21 days) than that of the Cvi allele (13 days) (Alonso-Blanco et al. 2003). Comparison of such data is hampered by the fact that experiments were not conducted at the same time.

The significance of co-location and candidate genes

Co-location of QTLs for different traits might be a first indication that the locus has pleiotropic effects on these traits, due to a common mechanistic basis. In some cases these pleiotropic effects were unexpected as found for the circadian period length (Swarup et al., 1999) and water use efficiency (McKay et al., 2003). These effects were associated with the *FLC*

flowering time locus and could be confirmed by studying mutants at this locus. Since it is assumed that seed longevity is determined by an overall tolerance to various seed stresses we expected, and found evidence for, co-locations for CDT QTLs and QTLs for tolerance to other seed stresses. However, in some cases co-locations were not found, indicating that tolerance to each specific stress has its own genetic basis. For germination-related traits it should be taken into account that dormancy might affect the data. However, no co-location between stress and dormancy QTLs was observed except on chromosomes 3 and 5 where salt tolerance and dormancy QTLs co-locate in opposite directions. The ability of QTL approaches to uncover novel loci determining stress resistance traits is also demonstrated in the case of heat tolerance. No QTL for this trait mapped to the bottom of chromosome 1, the location of the Hsp101 gene, which when mutated leads to severe heat sensitivity of imbibed seeds (Hong and Vierling, 2000).

Reactive oxygen species as a common factor

Co-location of QTLs related to germination under stressful conditions was observed on top of chromosome 1, where the Sha allele confers a higher tolerance to CDT survival, germination under saline conditions and germination after heat treatment. A common factor in all these stresses could be the release of ROS. For CDT it is known that seed deterioration can occur through the generation of oxidative stress (Khan et al., 1996). Saline conditions are also known to generate ROS (reviewed by Xiong and Zhu, 2002). Stressing seeds might also induce the expression of LEA proteins and after heat shock sHSPs are specifically expressed (Wehmeyer and Vierling, 2000). Besides acting as protector in preventing irreversible protein denaturation, sHSPs may modulate cellular redox state (Grene, 2002). This and the localization of several known ROS scavenging enzymes in this region, viz., catalase (Frugoli et al. 1996) and a superoxide dismutase (Kliebenstein et al., 1998) might point towards the production of active oxygen species as a mechanism in seed deterioration. Several other stress related genes were mapped in this region e.g. genes related to freezing tolerance, possibly also related to oxidative stress (Thorlby et al., 1999) and genes involved in drought stress, one of them having homology to a glutathione-S-transferase, an enzyme involved in reactive oxygen scavenging (Taji et al., 1999). However, germination on H_2O_2 could not confirm the relationship between ROS and germination. It might be expected that seeds with a higher tolerance to endogenously produced ROS might be more tolerant to exogenously applied H_2O_2. Therefore a QTL for H_2O_2 tolerance might be expected at this position on chromosome 1, but was not be detected. However, the transgression toward higher values indicates that there are more loci involved in H_2O_2 resistance than the one detected on chromosome 3. Alternatively, applying H_2O_2 might not reveal all types of ROS

tolerance, and hence not reveal all loci involved in ROS scavenging. The presence of a QTL for speed of germination, on the top of chromosome 1, might indicate that this locus is involved in germination in general and is not specific for germination under stress conditions, as was shown in tomato (Foolad et al., 1999) and *Brassica* (Bettey et al., 2000). A specific control of ROS during germination by this locus cannot be excluded because in barley (Bethke and Jones, 2001; Fath et al., 2001) and radish (Schopfer et al., 2001) it was shown that germination is accompanied by an increase of ROS, although our data did not provide arguments for this. The absence of co-location of a QTL for germination on H_2O_2 with any of the other stresses might be due to the fact that this locus is only involved in the metabolism of applied H_2O_2 and not involved in scavenging of endogenous H_2O_2.

The relation between inhibition of germination and dormancy

QTLs related to seed dormancy and germination on NaCl co-locate at chromosomes 3 and 5. Co-location of QTLs found for germination under salt stress and for dormancy was also observed in a barley (*Hordeum vulgare*) mapping population. In the Steptoe/Morex barley lines, dormancy on chromosome 7(5H), is conferred by the Steptoe allele (Gao et al., 2003) and the salt tolerance is conferred by the Morex allele (Mano and Takeda, 1997). This is similar in the L*er*/Sha population; the more dormant alleles have increased sensitivity to NaCl (Sha/Steptoe), while the less dormant alleles are more resistant to NaCl (L*er*/Morex). Since at these loci salt germination correlates with increased dormancy, these pleiotropic effects might be a consequence of the fact that seeds with a high germination potential (less dormant) might allow germination at higher osmotic values. A common factor in these processes might be the plant hormone ABA, which is involved in dormancy regulation. High salt conditions induce high levels of ABA and inhibit germination (Xiong and Zhu, 2002). The observation that non-dormant, ABA deficient mutants (Léon-Kloosterziel et al., 1996b) and ABA insensitive mutants (Queseda et al., 2000) can germinate at higher salt concentrations compared to wild-type also suggests a role for ABA. We did not obtain indications that ABA sensitivity per se, was involved in this population since no QTLs for ABA sensitivity co-located at these loci. However, there are mutants showing an altered response to applied ABA, *abi4* and *abi5*, without an altered dormancy (Finkelstein, 1994) and not all *reduced dormancy* (*rdo*) mutants have an altered ABA sensitivity (Peeters et al., 2002). Mano and Takeda (1997) suggested a role for ABA in germination under saline conditions in barley doubled haploid lines. They could only find co-location of QTLs for ABA and salt response in a population of Harrington/TR306 barley doubled haploid lines, while in the Steptoe/Morex doubled haploid lines no co-location could be found. We could not confirm this relationship between salt and dormancy by locating QTLs for ABA response at similar positions.

The only significant QTL for ABA sensitivity at the top of chromosome 3 co-locates with the pleiotropic seed stress locus (D_{50}) and better germination on ABA correlates with better germination in other stress conditions. This suggests that this locus affects germination in many conditions, which might be illustrated by a QTL for germination speed. A similar co-location of germination under various stresses and germination speed is observed on top of chromosome 1.

Regulation of germination, sugars and longevity

On top of chromosome 3, QTLs for germination under stress and non-stress condition co-locate. Mapping QTLs for germination speed could indicate a locus involved in the regulation of germination as such, as suggested by Foolad et al. (1999) and Bettey et al. (2000). The presence of a QTL for sucrose could also point in this direction since sucrose is probably the first metabolite involved in the initial germination process (Bewley and Black, 1994). Therefore, high endogenous sucrose levels may enhance germination even under stress conditions. Breakdown of lipids to sugars is not essential for germination as was shown by germination of seeds lacking the glyoxylate cycle but is essential for proper seedling establishment (Eastmond et al., 2000). However, the presence of a QTL for both CDT and natural aging might point toward an essential role for sucrose in seed longevity. Sucrose and stachyose are often associated with desiccation tolerance and seed longevity (Obendorf, 1997; Sinniah et al., 1998; Bailly et al., 2001). In a comparison of the QTLs obtained for seed longevity, by CDT survival, and for seed oligosaccharide (OS) contents it was already shown by Bentsink et al. (2000) that the variation observed for OS content does not clearly affect seed storability. The L*er*/Sha population does not rule out the possibility of a protective role for OS in seed longevity although only a very low amount of the total variance for seed sugar content could be explained. Sinniah et al. (1998) found that acquiring desiccation tolerance and seed longevity are separate processes, sucrose content could not be correlated to desiccation tolerance, but it could be correlated with potential seed longevity. In the L*er*/Sha RIL the latter correlation can be seen on top of chromosome 3 were a higher sucrose content from Sha alleles co-locates with a higher CDT survival and better germination under salt stress. In both cases Sha alleles conferred a higher tolerance. This co-location of QTLs for CDT survival and higher sucrose content on chromosome 3 was also seen in the L*er*/Cvi population (Bentsink et al. 2000). A possible explanation might be the protection of membranes by sucrose in the desiccated seeds due to water replacement (Hoekstra et al., 2001).

According to Sinniah et al. (1998), stachyose is possibly involved in both tolerance to desiccation and potential longevity. However, this relationship was not observed in the population described here, since a co-location on top of chromosome 4 is in opposite direction and the QTL for stachyose on chromosome 1 does not co-locate with a QTL for seed germination under stress conditions. The ratio of OS to sucrose could not be associated with a QTL for seed storability as was suggested by Obendorf (1997) although variation for this trait is limited in this population.

Which components of seed longevity are assayed by the CDT ?

Controlled deterioration tests simulate aging of seeds under controlled, but artificial, conditions and can be used to predict seeds storage potential (Hampton and TeKrony et al., 1995). Bentsink et al. (2000) showed the usefulness of the CDT to predict longevity since one QTL for CDT survival co-located with a QTL for naturally aged L*er*/Cvi seeds. This was confirmed in the L*er*/Sha RiI population by a similar co-location on top of chromosome 3. CDT reveals four loci in L*er*/Cvi (Bentsink et al., 2000) and three in L*er*/Sha, while natural aging only shows one in both populations. However, in both populations the range of germination percentages, after natural aging, found in the RILs is still above 60% indicating that finding QTLs for natural aging probably requires storage periods longer then 20 months for this batch of L*er*/Sha RIL seeds and longer than 4 years in L*er*/Cvi seeds used. The co-location of the CDT QTL on the top of chromosome 1 with QTLs for germination on salt and after a heat treatment indicates that one aspect of seed longevity is a general tolerance to stress. However, seed longevity might also be affected by genes that control others aspects like the amount of sucrose (top chromosome 3), which was found to co-locate with QTLs for germination on NaCl and CDT survival. The co-location of CDT survival and sucrose was also observed in the L*er*/Cvi population (Bentsink et al., 2000).

The QTL mapping approach appears to be a valuable method in elucidating the genetics, but also the physiological background of traits involved in seed quality. Further analysis such as fine mapping and the study of mutants of candidate genes will be needed to prove the pleiotropic effects that are now only suggested by co-locations of QTLs.

Acknowledgements

We would like to thank Elena Matteucci, Sohaee Negar and Corrie Hanhart for performing the salt germination assay and Shannon Parrington for the heat-stress germination test. Olivier Loudet and Carlos Alonso-Blanco for providing marker information before publication, Leónie Bentsink for instructions about the MapQTL program and Steve Tonsor and the STW supervision committee for useful suggestions and discussions. This research was funded by STW, project WBI4737 (E.J.M.C., G.J.R., H.B-V) the NATURAL program of the EU (QLG2-CT-2001-01097) and by a fellowship from the Government of Egypt to M. E-L. E.V. was supported by the National Science Foundation (POWRE grant), the Guggenheim Foundation and the ASDA (NRICGP)

Materials and Methods

Genotypes and culture conditions

A new RIL population was obtained from a cross between the accessions Landsberg *erecta* (L*er* - NW20) and Shakdara (Sha - N929) (pollen parent). The population of 114 RIL's was obtained via selfing and single seed descent from individual F_2 plants. F_9 seeds were sown in Petri-dishes on water-saturated filter paper and incubated at 4°C for 4 days and then transferred to a growth chamber at 25 °C. After 2 days of incubation, germinated seeds were transferred to soil and cultivated in an air-conditioned greenhouse (18°- 23°C) in a 16 h photoperiod. Two randomized plots containing 6 plants per lines were grown and seeds were bulked from 5 plants per line. The sixth plant was used to isolate DNA and seeds were harvested separately. Seeds were stored under ambient conditions until further use. To reduce the developmental and environmental effects on seed characteristics, the onset of flowering was synchronized. For that the late flowering RILs were planted two weeks before the entire set of 114 lines was planted again. The lines and marker data will be made available through the Arabidopsis stock centers.

DNA isolation and genotyping

DNA was isolated from greenhouse grown plants, one plant per line per plot. The Bernatzky and Tanksley (1986) protocol was adapted for rapid extraction of small quantities. Flower buds were harvested in liquid nitrogen and ground in 330µl of a preheated (65°C) extraction solution (125µl extraction buffer (0.35 M sorbitol. 100 mM Tris, 5 mM EDTA, pH 7.5 (HCl) together with 175 µl lysis buffer (200 mM Tris, 50 mM EDTA, 2 M NaCl, 2 % (w/v) cetyl-trimethyl-ammonium bromide) to which 30 µl sarkosyl (10% w/v)) was added. The mixture of crude plant material and extraction solution was incubated for 30 minutes at 65°C during this period occasional shaking was applied. Hereafter a solution of 400 µl chloroform/isoamyl alcohol (24:1) was added and vortexed. After centrifuging for 5 minutes at maximum speed in an Eppendorf centrifuge the water phase was transferred to a new tube. An equal amount of cold isopropanol was added to precipitate the DNA by carefully inverting the tube several times. After 10 min centrifugation at maximum speed in an Eppendorf centrifuge the water-alcohol mixture was discarded and the pellet washed with 70% cold ethanol. The pellet was left to dry and dissolved in water containing RNAse A and incubated 30 minutes at 37°C. Thereafter it was stored at 4°C. If the genotyping required more DNA; this was isolated from seedlings grown on half strength MS media containing 10 % (w/v) sucrose from seeds that had been harvested separately. DNA was now isolated using the Wizard ® magnetic 96 (Promega; #FF3760) DNA isolation kit.

Genotyping was done on F9 plants using microsatellite and CAPS markers. The CAPS and microsatellite markers were either found in the Tair database (http://www.arabidopsis.org) or taken from http://www.inra.fr/qtlat/msat. Primers for markers that cannot be found in these two databases are summarized in Table 5.4. The *ADH* CAPS marker polymorphism was detected with the enzyme XbaI. All markers were first checked for polymorphism between L*er* and Sha thereafter the polymorphic

markers were used to genotype the all individual RIL lines. For the microsatellite markers a standard protocol of 30s 94°C, 30s 50°C and 30s 72°C (35 cycles) was used except for FRI (54 degrees, 1 minute extension) and FLC (52 degrees annealing, 2 minutes extension).

Table 5.4: New markers used in genotyping the L*er*/Sha RIL population

chr	marker name	BAC	primer 1 (5' to 3')	primer 2 (5' to 3')	origin
1	F3F19	F3F19	CGTCCAGACACTGACATTGGTTTTAGG	CCACTCACGTTCAGTGGGGTTAAACT	Genetics
1	F6D8-94	F6D8	GTCATTGGTTGCAATACGAGAGC	GCTGCCTCTTCCTTGTAAAGCC	Indel
2	F3P11-6b	F3P11	TTCAATCTTCTCTACTGTCTTCG	AGCAGGAAGTAGTAAGTGGAATA	Alonso Blanco
3	F8J2	F8J2	GTGACCCAAGTGGGATCTCTC	ACGTGTGGGCAATCTTTTAAT	Genetics
4	FRI	F6N23	CATGTCGTAATCATGCAACC	GAAGATCATCGAATTGGC	Tony Gendall
4	C6L9-78	C6L9	TGCTTTGTGAAAGTCTCTCATGCC	CCCTTTGATTGCTCAGTGATATCG	Indel
4	M4I22-22	M4I22	CGCTTTTAGGGGTAATATCGTCAC	CTGTGTGATCAGGCAAAACCAGT	Indel
5	FLC	T31P16	CATTGGATAACTAATCTTTGAGC	CAGGCTGGAGAGATGACAAAA	Tony Gendall
5	K9D7	K9D7	GCTGTTGTAATTTGTGATAGGG	CATATGCCACGTTTCTTAATAG	Genetics
5	K8A10	K8A10	AATGCCAAGGATCAAAAGTGTT	GATGATCGGAGGAAAATGAAAA	Genetics

Genetics: primers developed by co-workers in genetics department.
Indel: primers developed based on L*er* and Col indel as found in the Cereon database (Jander et al., 2002).
Alonso Blanco: primers were a gift from Dr. Alonso Blanco.
Tony Gendall: primers were a gift from Dr. Tony Gendall (Gazzani et al., 2003).

Map construction and QTL analysis

A set of 66 markers covering most of the Arabidopsis genetic map at intervals of 1 to 15 cM was constructed with the Joinmap program (version 3.0, Plant Research International, Wageningen, The Netherlands). The computer program MapQTL (version 4.0, Plant Research International, Wageningen, The Netherlands) was used to identify and locate QTL linked to molecular markers using both interval mapping and multiple-QTL model mapping (MQM) methods as described in its reference manual (http://www.plant.wageningen-ur.nl/products/). The estimated additive effect and the percentage of variance explained by each QTL as well as the total variance explained by all of the QTLs affecting a trait were obtained with MapQTL in the final MQM model. For this, different co-factor markers were tested around a putative QTL position (van Ooijen and Maliepaard, 1996) selecting as final co-factors the closest marker to each QTL i.e. those maximizing the LOD score. A LOD threshold of 2.6 was applied to declare the presence of a QTL. We verified this threshold for interval mapping by applying the permutation test to each data set (10 000 repetitions) and found a $P = 0.05$ LOD varying between 2.4 and 2.6. Two-LOD supports intervals were established as 95% confidence intervals (van Ooijen, 1992). For every trait, two-way QTL interactions were analyzed at a significance level of $P < 0.05$, using the general linear model module of the statistical package SPSS version 11.0.1 (SPSS, inc, Chicago). For each analysis, the closest linked markers to the corresponding detected QTL were used as random factors in the ANOVA (the same markers used as co-factors in the MQM mapping MapQTL). Heritability as the proportion of the variance explained by the between line effects was calculated using the general linear model module of the statistical package of SPSS version 11.0.1 (SPSS, inc, Chicago). The QTL effect was calculated using the untransformed data, the value was obtained by fixing the same co-factors as with the transformed data used to determine the positions of the QTLs.

Sugar content measurement

One hundred seeds from bulks of 5 plants of one replicate were weighed. Sugars were extracted from these 100 seeds by heating for 15 minutes at 76°C in 80% (v/v) methanol with the addition of 25 µg melezitose as internal standard. After heating the homogenate was centrifuged for 5 min at 10 000g. The supernatant was vacuum-evaporated and its residue resuspended in 0.5 ml pure water and injected into a Dionex HPLC system (Dionex Corporation, Sunnyvale, CA). Sugar content was determined with a high-pH-anion-exchange HPLC, using a gradient pump module (model GP40) and an ED40 pulsed electrochemical detector (Dionex Corporation, Sunnyvale, CA). Sugars were chromatographed on a CarboPac PA100 4- X 250-mm column (Dionex) preceded by a guard column (CarboPac PA100, 4 X 50 mm). Mono-, di, and trisaccharides were separated by elution in increasing concentration of NaOH (50-200 mM), with a flow rate of 1 ml per minute. Peaks were identified by co-elution of standards. Sugar quantity was corrected by means of the internal standard and transformed to micrograms of sugar per milligram of seed.

Germination assays

To assess seed dormancy, two replicas of 50 to 100 seeds were sown on water-saturated filter paper in Petri-dishes. Germination was scored after 7-d incubation in a growth chamber (25°C, 16 h light period). All the lines were sown at several intervals after harvest until the germination was between 95-100%; meanwhile seeds were stored under dry conditions. Seed dormancy of a genotype was estimated in a single parameter as the number of days of seed dry storage required to reach 50% germination ($DSDS_{50}$). To estimate the $DSDS_{50}$ value of each genotype, all the measurements of germination proportions at various times during seed storage were used for probit regression on a logarithm time scale applying the regression module of the statistical package SPSS version 11.0.1 (SPSS, inc, Chicago) (Alonso-Blanco et al., 2003). The $DSDS_{50}$ values were individually assessed for all RILs and each replicate Thereafter, the $DSDS_{50}$ values were averaged and QTL analysis was performed with this average value.

Germination assays were performed, in duplicates, with seeds bulked from 5 plants. These bulks were harvested from two greenhouse grown replicates. Prior to the transfer to the growth chamber (25°C, 16 h light period) seeds were stored for 4 days at 4°C unless stated differently.

Sodium chloride and mannitol tolerance was estimated by germinating 50 to 80 vapor sterilized seeds on water agar containing 150 mM of NaCl or 400mM of mannitol. Plates were stored for 3 days at 4°C. Germination was counted after 13 days (NaCl) or 7 days (mannitol). The germination data were corrected for germination on medium containing no salt and then probit transformed for each line and both replicates. The average value was used for QTL analysis.

Vapor seed sterilization of seeds was done by placing seeds in an opened Eppendorf tubes in a desiccator jar. Then a 250 ml beaker containing 100 ml commercial bleach was placed inside and 3 ml concentrated HCl was added. The desiccator jar was closed and the seeds were sterilized by chlorine gas. After 2 to 3 h the jar was opened and the Eppendorf tubes were closed until use.

Tolerance to hydrogenperoxide or ABA was estimated by germinating 50 to 80 seeds on filter paper either saturated with 0.6% (v/v) H_2O_2 or 1 µM ABA. Plates were stored for 7 days (only ABA) at 4°C and final germination was counted after 7 days. The germination percentages per duplicate were first averaged then corrected for germination at day 7 on water and thereafter probit transformed for each line and both replicates. These values were then averaged and used for QTL analysis.

Natural aging was determined by germinating 50-80 seeds of seed lots that had been stored for 20 months at ambient conditions on water saturated filter paper. Final germination was counted after 7 days. The germination percentages per duplicate were first averaged, thereafter both replicates were probit transformed and averaged to perform the QTL analysis.

Speed of germination was determined by germinating 50-80 seeds on water-saturated filter paper. Germination was first determined at 36 hours after transfer to the light, a second germination percentage was determined after 60 hours and final germination was determined after 7 days. Both duplicates for each replicate were first corrected for germination at day 7 on water, thereafter these corrected germination percentages for 36 and 60 hours were averaged per duplicate. This average germination percentage was then probit transformed for each duplicate; these were averaged within the greenhouse grown replicate and thereafter both replicates were averaged to perform the QTL analysis.

Controlled deterioration tests

Controlled deterioration tests were performed according to Tesnier et al. (2002). Briefly, seeds are equilibrated at 85% relative humidity (15°C) and day 0 controls are immediately dried back at 32% relative humidity. Treatment is done by storing the seeds (at 85% RH) for a number of days at 40°C (2, 4 and 6 days). Then these seeds are also dried back at 32% RH (20°C) and stored at 4°C until the germination assay was performed. Two replicates of 50 seeds were tested for each day of treatment. Seed deterioration was estimated as a single parameter as the number of days of treatment required to reach 50% germination (D_{50}). Germination proportions of all treatments were used for probit regression on a time scale, in days, applying the regression module of the statistical package SPSS version 11.0.1. The D_{50} points of both replicates were then log transformed, to improve normality, averaged, and used in QTL mapping procedures.

Heat Assay

Seeds, harvested from plants grown in a climatized greenhouse in Tuscon (Arizona, USA) were sown on water-saturated filter paper. Thereafter they were left to imbibe for 18 H at room temperature, transferred to 50°C and left for 8 H as heat treatment. The germination percentage was determined after 7 days. This was done in three replicates of each line. To normalize the data all germination percentages were probit transformed and QTL mapping was with the average probit.

Chapter 6

Summarizing discussion

Summarizing Discussion

Modern crop production systems require a high degree of precision in crop establishment. Consequently seeds of high quality are required that will consistently produce viable and vigorous seedlings without defects after storage, even under sub-optimal growth conditions. Seeds can differ considerably in their capacity to produce vigorous seedlings. The quality of seeds is determined by many factors, starting with seed development and seed maturation on the mother plant. After seed harvest the storage conditions play a vital role. Orthodox, desiccation tolerant seeds stored at low relative humidity's (RH) and low temperature can remain viable for many years. Non-viable seeds are the result of accumulating damage occurring during storage, which can be sustained by all organs. Desiccated seeds are equipped with several protective mechanisms against damage, which have often been associated with the prevention of the occurrence and damage by reactive oxygen species (ROS). These include the down regulation or prevention of metabolism (dormancy, glassy matrix, low moisture content) and the presence of antioxidants, counteracting the negative effects of ROS or ROS production.

The main aim of the work presented in this thesis was to investigate the genetic regulation of seed quality tested as seed longevity. Since seeds can remain viable for many years in the proper environment, aging of seeds was simulated by a controlled deterioration test (CDT). Furthermore several other techniques like conductivity measurements and germination under stress conditions, possibly correlated to seed deterioration, were applied for the analysis of seed quality. We used Arabidopsis since it is suitable for genetic and molecular research and because many resources are available. Longevity of seeds was tested for existing mutants that might be affected in seed storability and new mutants were isolated as enhancers or suppressors of the *abscisic acid insensitive3* (*abi3*) mutants. In addition, natural variation present among Arabidopsis accessions was exploited for the identification of loci that either directly, or as modifiers of poorly storable mutants, play a role in seed storability.

Genetic factors involved in seed longevity

Seed longevity depends on the avoidance of damage, which can be due to mechanisms that damage the integrity of essential parts of seeds such as membranes, proteins and DNA. Although such damage may occur in a dry metabolically inactive state it can be assumed that when metabolism is present, even at a very low level, this will influence the amount of accumulating damage. The metabolic state is influenced by environmental conditions but might also be affected by the state of quiescence.

In Chapter 2 we showed that mutants having a reduced primary dormancy, like *abi3* and *aberrant testa shape* (*ats*), showed a decrease in longevity. A possible explanation might be that non-metabolizing dormant seeds start later with the accumulation of detrimental products. Therefore at a given point in time, compared to non-dormant seeds of the same age, the dormant seeds might have accumulated less damage resulting in enhanced seed storability. A specific role for abscisic acid (ABA) in this process cannot be excluded.

The detrimental products receiving most attention in relation to seed longevity in literature are ROS (Hendry, 1993: McDonald, 1999). ROS are among others the superoxide and hydroxyl molecules and, although not a real radical, hydrogen peroxide (H_2O_2), since H_2O_2 can provide, through the Fenton reaction, again hydroxyl molecules. Especially in oil containing seeds, like Arabidopsis, ROS may be an important factor since they can be generated through lipid peroxidation. There is a vast network of antioxidants to dispose of the ROS, like ascorbic acid (vitamin C), glutathion, flavonoids and the enzyme catalase. The relative amount of these anti-oxidants has often been correlated with seed longevity. The genetic approach described in this thesis used mutants with deficiencies in their antioxidant system, like *vitamin C deficient1-1* (*vtc1-1*; Conklin et al., 1996), *cadmium sensitive2-1* (*cad2-1*: glutathion deficient; Howden et al., 1995) and *catalase1 catalase3* (*cat1 cat3*), which has a deletion in two of the three catalase genes present in Arabidopsis (Salomé and McClung, 2002). Since redundancy in scavenging pathways was expected, also “double” mutants were studied. However, neither the single mutants nor the “double” antioxidant deficiency mutants showed a large reduction, in their tolerance to a CDT or when germinated in the presence of H_2O_2, compared to their respective controls (Chapter 2). Three possible explanations for this limited effect are that firstly: ROS accumulation and the level of antioxidants is of minor importance in generating damage in seeds during CDT conditions. Secondly: the mutant combinations studied still have antioxidant levels far above the critical thresholds. This possibility cannot be excluded since the antioxidant levels have not been analyzed in the mutant seeds. Thirdly: other protection mechanisms might be equally, or even more, important. These may be other antioxidant systems such as α-tocopherol, superoxide dismutase (SOD) (reviewed by Grene, 2002) and peroxiredoxin (Haslekas et al., 1998). In addition structural factors are important, such as the effectiveness by which membranes and other macromolecules are protected probably by the successful formation of a biological glass (reviewed by Hoekstra et al., 2001 and Oliver et al., 2001).

Testa mutants like *ats* and *transparent testa4-1* (*tt4-1*) and “double” mutants between them and mutants with deficiencies in antioxidant scavenging showed a decrease in germination when imbibed at H_2O_2. Furthermore, the *ats* mutant showed a strong decrease in germination after a four year storage (Chapter 2). It was shown before that the seed coat

of these mutants is more permeable (Debeaujon et al., 2000) and indicates the necessity of a "functional" seed coat for proper seed longevity.

The study of a complex trait, like seed storability, by mutagenesis is complicated since several processes, each with their own genetic basis contribute to the trait. In addition, pathway as well as gene redundancy is relatively frequent in Arabidopsis (AGI, 2000). These aspects make that often only a combination of single mutants will reveal the role of the respective genes in a particular process. We used *abi3* mutants, for which an allelic series is available with different levels of maturation defectiveness (Wolkers et al., 1998; Bies-Ethève et al., 1999). The many pleiotropic effects of the *abi3* mutants indicate that the ABI3 protein affects many processes during seed maturation and germination, including the storability of seeds. These *abi3* mutants provided the possibility to select both enhancers and suppressors.

The *abi3-1* mutant can be stored for at least one year under ambient conditions before it loses viability; enhancers of the *abi3-1* phenotype would shorten the storability. An enhancer of the *abi3-1* phenotype was identified on the basis of its greener seeds in *abi3-1* background; hence it was named *green seed* (*grs*). The *grs* single mutant was not green seeded. The gene was located on chromosome 4 between the markers nga8 and DET1. In *abi3-1* background the *grs* mutation affected neither the ABA phenotype nor dormancy but it did affect seed color and storability. The specific effect on seed longevity was confirmed with the single *grs* mutant, which showed a decrease in storability compared to wild-type seeds in both a CDT and after a prolonged storage at elevated RH (60%). The decrease in viability and vigor could be related to membrane damage since it was shown that conductivity increased while germination decreased. During seed maturation, ABI3 regulates several processes: acquiring dormancy and long-term storability and the loss of chlorophyll. Hypothesized was that *GRS* is a common regulator in the latter two processes but not of dormancy (Chapter 3).

A mutagenesis experiment using the most severe *abi3* mutant (*abi3-5*) as parental material resulted in four novel suppressor mutants (Chapter 4). These mutants were isolated based on their prolonged longevity in *abi3-5* background, hence they suppressed the *abi3-5* storability phenotype and were named *suppressor of abi* (*sua*). The four mutations represented four independent loci as was concluded from their map-position (Fig 6.1) and allelism tests. Some of these mutants show an altered response when germinated on ABA, however dormancy was unaffected. How these mutations act in wild-type background is currently not known (Chapter 4). These mutant screens have not been saturating since independent mutant alleles have not yet been found. The relatively small mapping populations and the variable and quantitative expression of the trait in general, made it difficult to obtain accurate map positions.

Naturally occurring populations provide another source of genetic variation for seed storability. This was also indicated by the experiments with some “double” mutants carrying oxidative stress scavenging defects. These “double” mutants showed a different response to CDT and germination on H_2O_2 depending on their genetic background. Allelic differences between accessions were observed before when quantitative traits loci (QTL) for CDT survival were mapped in the Landsberg *erecta* (L*er*)/Cape verde islands (Cvi) recombinant inbred line (RIL) population (Bentsink et al., 2000). Accession differences in response to oxidative stress, induced by paraquat, were observed by Abarca et al. (2001) who found that the accession Cvi was more tolerant to this treatment than the other accessions tested (L*er* and Colombia).

This naturally occurring genetic variation was exploited in two ways, firstly by selecting modifiers of the *abi3-5* and *leafy cotyledon1-3* (*lec1-3*) mutants (Chapter 4), using them as a “sensitive” background (Matin and Nadeau, 2001) for selection, and secondly by subjecting seeds of a new RIL population to several stresses (Chapter 5). The *lec1-3* mutant is like the *abi3-5* mutant severely disturbed in its seed maturation and has a strongly reduced storability.

Modifiers, originating from natural variation, could be identified in the progeny of crosses between 20 accessions and both the *abi3-5* and the *lec1-3* mutant. Starting in the F2 generation, *abi3-5* and *lec1-3* mutants that showed prolonged seed longevity were selected. This selection provided in total eight modifiers: three from selection in *lec1-3* background and five from selection in *abi3-5* background. The genetic characterization of these modifiers provided a limited number of map positions (Fig 6.1). Furthermore it was shown that during storage of the *lec1-3* modifier lines the conductivity increased, indicating the accumulation of membrane damage. This increasing amount of electrolyte leakage was also observed in the *grs* mutant, where poor storability was correlated with high conductivity (Chapter 3). The isolation of *lec1-3* modifiers in a wild-type background did not improve the survival of these lines in a CDT in comparison to the respective wild-types from which they originated. These observations suggest that the modifier does not directly influence the target for acquiring longevity, but affects steps in the pathway leading towards seed longevity. Possibly this may include the development of desiccation tolerance in seeds, which is a prerequisite for seed longevity.

In Chapter 5 we describe the use of natural variation in a new RIL population derived from a cross of L*er* x Shakdara (Sha). These accessions were used because preliminary tests had shown that Sha was very resistant to stress applied during germination. This new set of 114 F9 RILs was genotyped with 65 PCR based markers and the visible marker *erecta*. In this RIL population we identified a number of loci for traits related to seed quality. For dormancy expressed as the number of days of seed dry storage required to reach 50%

germination ($DSDS_{50}$) four loci were identified. Seed sugar content revealed in total five loci, one for sucrose, two for stachyose and two for the ratio of oligo saccharides to sucrose. Germination after applied stress tested as tolerance to CDT, heat, salt (NaCl), mannitol, H_2O_2 and ABA, revealed in total 13 QTLs. Natural aging revealed one QTL and unstressed speed of germination identified two QTLs. Some of these loci for different traits co-located and might point towards a common genetic basis. In most cases Sha alleles conferred to a higher tolerance to the applied stresses. The Sha alleles also increased dormancy, prolonged longevity and increased speed of germination.

Figure 6.1 summarizes all mutations, modifiers and QTLs known to influence seed quality. These loci were either found in literature or have been identified in this thesis and show the genetic complexity of seed quality.

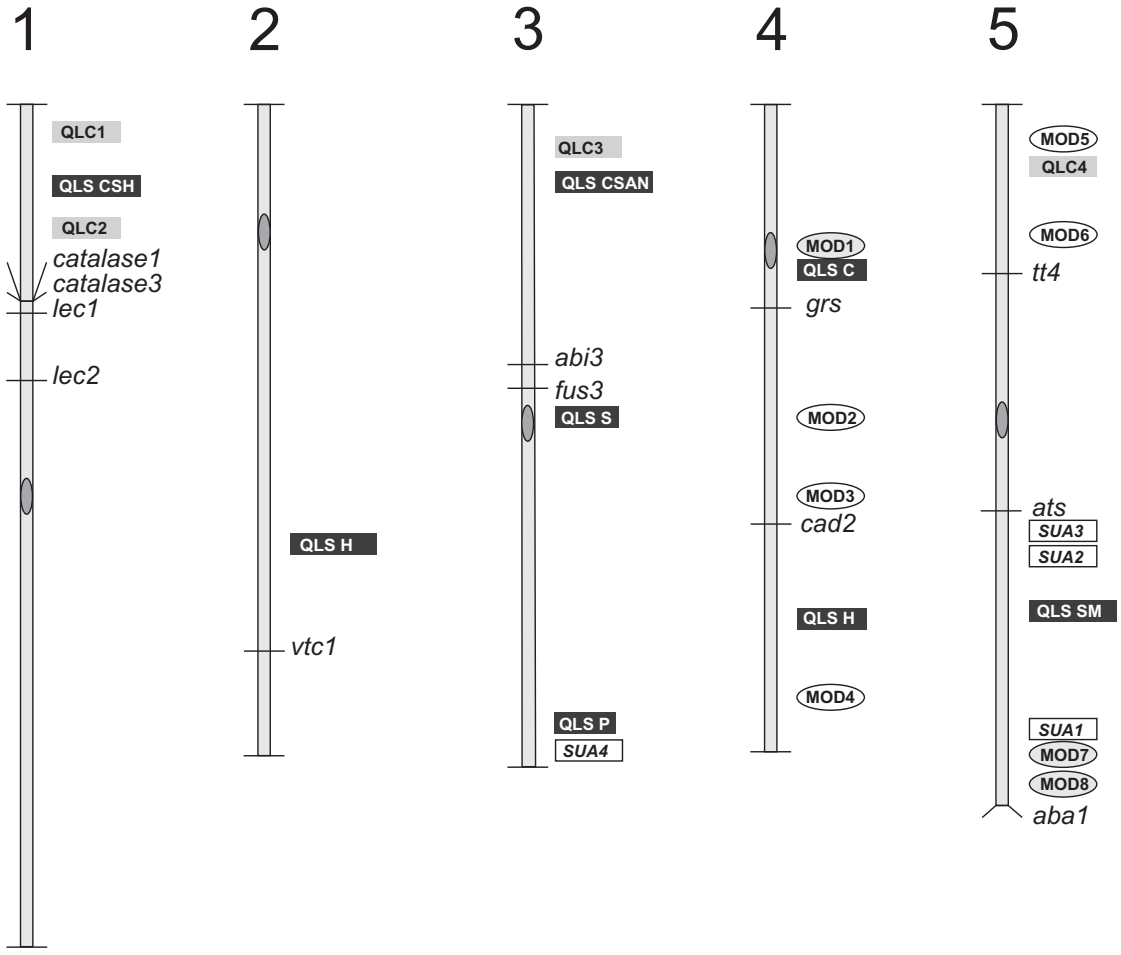

Figure 6.1: Schematic view of the Arabidopsis genome showing the mutant loci, modifiers and polymorphic QTLs affecting seed quality. Mutants are in italic, QTLs identified as a response to CDT in the L*er*/Cvi RIL population are in gray boxes (QLC) (Bentsink et al., 2000). Black boxes represent QTLs identified in the L*er*/Sha RIL population (QLS) (Chapter 5). The characters in the black boxes behind QLS indicate the type of QTL, C: QTL identified in response to CDT, S: in response to salt stress, H: in response to heat stress, M: in response to mannitol, A in response to ABA, P in response to H_2O_2 and N for natural aging. White boxes represent the *sua* mutants and the modifiers isolated from accession crosses are represented as ovals, modifiers identified in a cross with *lec1-3* are in gray ovals, whereas those from *abi3-5* are in white ovals.

The relevance of co-locations of seed quality loci

Figure 6.1 shows that some QTLs influencing germination and longevity co-locate with either modifiers and/or suppressor mutations. This could point towards the same genes although due to the crude map-positions and the use of different mapping populations for the individual loci, the possibility of different genes cannot be ruled out.

There are however two locations, the top of chromosome 1 and the top of chromosome 3, were QTLs for germination under stress conditions do not co-locate with newly identified modifiers or mutations. Therefore, it might be possible that these two locations do not contain genes involved in germination under stress but contain genes that regulate general aspects of germination also under non-stressed conditions. Loci affecting germination were identified by QTL mapping of germination speed, under non-stress conditions, in both tomato (Foolad et al., 1999) and *Brassica* (Bettey et al., 2000). Both authors argued that loci identified for faster germination were involved in regulation of germination as such, and not specific for germination under stress. We could identify QTLs for germination speed at these two positions, at the top of chromosome 1 and 3, indicating a general regulation of germination.

In both barley (Bethke and Jones, 2001; Fath et al., 2001) and radish (Schopfer et al., 2001) it was shown that germination is accompanied by an increase of ROS. Furthermore Puntarulo et al. (1991), showed an increase in both oxidants and antioxidants during germination of soybean and similar observations were made during germination in *Pinus pinea* L. (Tommasi et al. 2001), wheat (Cakmak et al. 1993) and *Zea mays* L. (Leprince et al., 1994). Furthermore, in the area on top of chromosome 1, we found QTLs for stressed and non-stressed germination co-locating with a large number of oxidative stress related genes (Kliebenstein et al., 1998; Taji et al., 1999; Thorlby et al., 1999). This might suggest that containment of ROS is important even during unstressed germination. However, ROS containment is also important during storage; therefore a protective role of these gene products during storage cannot be excluded.

The regulatory role in germination of the top of chromosome 3 might lie in the availability of sucrose, since also a QTL for sucrose content was identified in this region (Chapter 5). A similar co-location of a CDT QTL and a sucrose accumulation QTL was also observed in the L*er*/Cvi RIL population (Bentsink et al., 2000). Sucrose is probably the first storage product used in the initial germination processes (Bewley and Black, 1994). This is supported by the observation that low concentrations of exogenous sugars are able to relieve the inhibition of germination imposed by ABA (Garciarrubio et al., 1997; Finkelstein and Lynch, 2000). High exogenous levels of sugars are able to increase the internal ABA concentration thereby inhibiting germination (Price et al., 2003). We do not expect the same

effect of endogenous sugar levels since these are used to provide energy for germination (Pritchard et al., 2002). The non-dormant and fast germinating *abi3* mutants accumulate 3-6 fold more sucrose during seed development compared to wild-type (Ooms et al., 1993). Therefore seeds accumulating higher amounts of sucrose might show a higher germination rate, even under stress conditions. Breakdown of lipids to sugars is not essential for germination as was shown by germination of seeds lacking the glyoxylate cycle (Eastmond et al., 2000). However, proper seedling establishment and survival is dependent on lipid breakdown. Poor seedling establishment is also observed for *abi3-5* and *lec1-3* mutants lacking storage compounds. These observations, suggesting the involvement of sucrose in promoting germination, are supported by the presence of a QTL for germination speed at this position. One could imagine that high endogenous levels of sucrose enhance germination, indicating that this locus is always involved in regulation of germination. However, the presence of a QTL for natural aging at the top of chromosome 3 (Chapter 5) might point towards a role for sucrose in storability as well. Sucrose can act as water replacement providing membrane protection (Hoekstra et al., 2001). Membrane stability can be assessed by measuring seed leakage. In some of the identified lines with enhanced storability, *grs* and modifiers isolated in *lec1-3* background, we could show that there is a good correlation between storability and conductivity.

Since QTLs on the top of chromosome 1 and 3 did not co-locate with modifiers or newly identified mutants it is suggested that these loci are involved in different processes, such as the general regulation of germination. The location of a QTL for speed of germination seems to indicate this. However, as argued a role in protection of seeds during storage or during germination in stress conditions cannot be excluded. Therefore, the enhancers or suppressors selected might be specific for the function of both *lec1-3* and *abi3-5* or the processes that they influence directly such as acquiring desiccation tolerance. A different strategy, for instance selection using CDT performance or maybe a different “sensitive” genetic background, might be needed to select additional genes by mutant or modifier selection.

The QTLs, modifiers and mutants, especially those that co-locate require further characterization. Eventually cloning is necessary to establish whether these co-locations are due to pleiotropic effects or due to closely linked genes. It has been shown that pleiotropic effects that were not expected could be found by co-locating with QTLs for different traits. This is illustrated for the FLC flowering time locus, which also seems to control circadian period length (Swarup et al., 1998) and water use efficiency (McKay et al., 2003). This pleiotropism could be confirmed by studying mutants at this locus.

Seed longevity and the genetic factors involved

Seeds gradually loose their viability due to an accumulation of damage over time. For orthodox, desiccation tolerant seeds, it is predicted and shown that the decline can take hundreds of years, when seeds are stored under favorable conditions (Hay et al., 2003). Tolerance to desiccation is a survival mechanism adopted by orthodox seeds (Wilson Jr., 1995) and develops during seed maturation (Bewley and Black, 1994). During the last period of seed maturation, well beyond the moment desiccation tolerance has been acquired seeds gain in longevity (Hay et al., 1997; Jalink et al., 1998) indicating that these are, at least partially, distinct processes. Desiccation tolerance requires the proper protection of membranes and proteins in which sugars and LEAs are suggested to play an important role (Hoekstra et al., 2001). Curtailing the production of ROS is expected to enhance the survival, although we were not able to establish a relationship between mutants with a reduction antioxidant levels, due to a genetic defect, and seed longevity. Furthermore, it is clear that mutants with maturation defects like *lec1* and *abi3* produce very short-lived seeds. Modifiers of these mutations can be isolated to elucidate the genetic regulation of desiccation tolerance and indirectly or directly seed-longevity. Maternal genetic factors like seed coat formation start showing their defects during maturation but the real problem becomes evident after storage and during imbibition, when a higher permeability might cause problems. The processes and genetic factors described in this thesis are summarized in Figure 6.2.

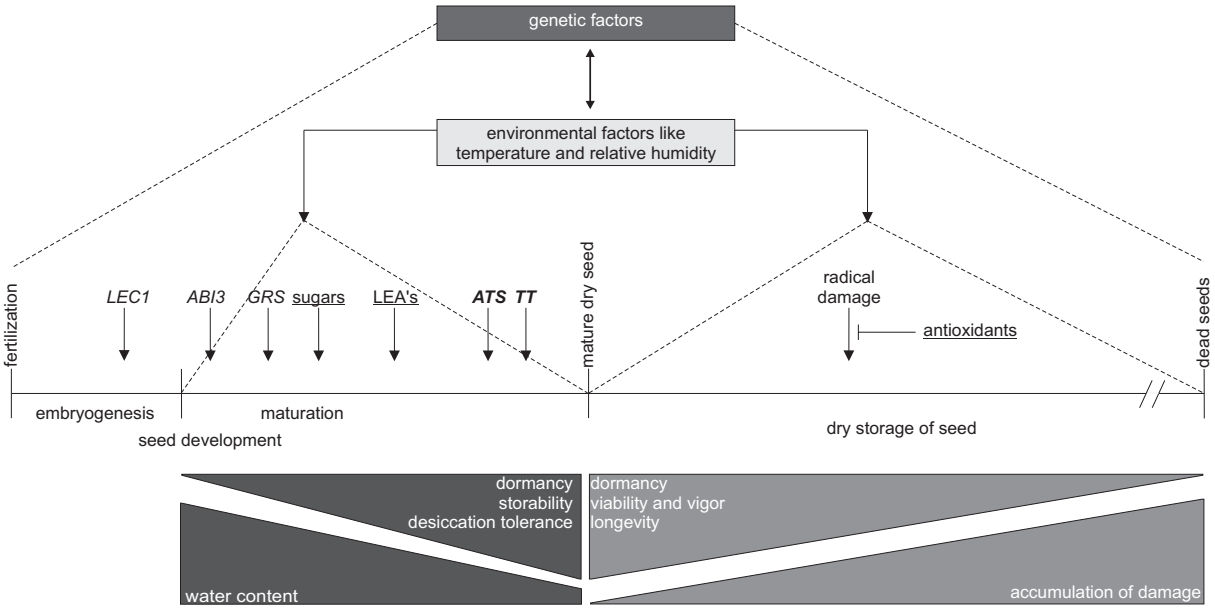

Figure 6.2: Diagram summarizing processes and genes involved in seed longevity. Genetic factors play a role during the whole life cycle of seeds and are influenced by environmental factors. Genes are displayed in italics; maternal genes are displayed in bold and italics. Underlined are compounds of which the expression is controlled by several genes.

We have demonstrated that many genes control seed quality; previously this was predicted by Lindstrom (1942) and Dickson (1980). Possible co-locations of QTLs, modifiers and mutations (Fig 6.1) might point towards the involvement of the same genes and would reduce the number of major players in seed longevity. However, many genes with small effects are probably missed by the techniques we used and this is partially due to the variable and quantitative expression of a trait as longevity. Although large effect mutations are not likely to be present in commercial crops, small effect natural allelic variation and mutations in genes affecting seed quality could be responsible for poorer seed quality in some of them. Seed quality traits have rarely received attention by breeders because it is a complex trait under polygenic control. Moreover, potential longevity problems are prevented by improved storage conditions at the seed companies. However when treatments that could reduce longevity such as priming are more frequently applied it might be desirable to pay more attention to this trait. Marker assisted breeding would be helpful when chromosomal locations and the allelic effects are determined.

Our analysis in Arabidopsis has shown that loci affecting seed longevity can be identified and for instance the analysis of such traits in Brassica has started (Bettey et al., 2000). Knowledge on the synteny between a crop like *Brassica* and Arabidopsis will allow the recognition of similar loci on the basis of map positions. It is likely that often similar genes may also play a role in less related crops as was shown convincingly for rice and Arabidopsis for another complex trait like flowering (Izawa et al., 2003). The next challenge will be to complete the identification of relevant loci for seed longevity and to understand the molecular function of these genes.

References

Aarts MGM, Corzaan P, Stiekema WJ, Pereira A (1995) A two-element enhancer-inhibitor transposon system in *Arabidopsis thaliana*. Mol Gen Genet 247: 555-564

Abarca D, Roldán M, Martín M, Sabater B (2001) *Arabidopsis thaliana* ecotype Cvi shows an increased tolerance to photo-oxidative stress and contains a new chloroplastic copper/zinc superoxide dismutase isoenzyme. J Exp Bot 52: 1417-1425

Alonso-Blanco C, Bentsink L, Hanhart CJ, Blankenstijn-de Vries H, Koornneef M (2003) Analysis of natural allelic variation at seed dormancy loci of *Arabidopsis thaliana*. Genetics 164: 711-729

Alonso-Blanco C, Koornneef M (2000) Naturally occurring variation in Arabidopsis: an under exploited resource for plant genetics. Trends Plant Sci 5: 22-29

Alonso-Blanco C, Blankenstijn-de Vries H, Hanhart CJ, Koornneef M (1999) Natural allelic variation at seed size loci in relation to other life history traits of *Arabidopsis thaliana*. Proc Natl Acad Sci USA 96: 4710-4717

Alonso-Blanco C, El-Assal SD, Coupland G, Koornneef M (1998a) Analysis of natural allelic variation at flowering time loci in the Landsberg *erecta* and Cape Verde islands ecotypes of *Arabidopsis thaliana*. Genetics 149: 749-764

Alonso-Blanco C, Peeters AJM, Koornneef M, Lister C, Dean C, van den Bosch N, Pot J, Kuiper MTR (1998b) Development of an AFLP based linkage map of Ler, Col and Cvi *Arabidopsis thaliana* ecotypes and construction of a Ler/Cvi recombinant inbred line population. Plant J 14: 259-271

Arabidopsis Genome Initiative (2000) Analysis of the gene sequence of the flowering plant *Arabidopsis thaliana*. Nature 408: 796-815

Association of Official Seed Analysts (1983) Seed vigour testing handbook. Contribution no. 32 to the handbook on seed testing.

Bailly C, Audigier C, Ladonne F, Wagner M-H, Coste F, Corbineau F, Côme D (2001) Changes in oligosaccharide content and antioxidant enzyme activities in developing bean seeds as related to acquisition of drying tolerance and seed quality. J Exp Bot 52: 701-708

Bailly C, Benamar A, Corbineau F, Come D (1997) Changes in superoxide dismutase, Catalase and glutathion reductase activities in sunflower seeds during accelerated ageing and subsequent priming. In RH Ellis, M Black, AJ Murdoch, TD Hong eds, Basic and applied aspects of seed biology. Kluwer academic Publishers, Dordrecht, pp 665-672

Bailly C, Benamar A, Corbineau F, Come D (1996) Changes in malondialdehyde content and in superoxide dismutase, catalase and glutathion reductase activities in sunflower seeds as related to accelerated ageing. Physiol Plant 97: 104-110

Baud S, Boutin J-P, Miquel M, Lepiniec L, Rochat C (2002) An integrated overview of seed development in *Arabidopsis thaliana* ecotype Ws. Plant Physiol Biochem 40: 151-160

Beaudoin N, Serizet C, Gosti F, Giraudat J (2000) Interactions between abscisic acid and ethylene signaling cascades. Plant Cell 12: 1103-1115

Bentsink L (2002) Genetic analysis of seed dormancy and seed composition in *Arabidopsis thaliana* using natural variation. PhD thesis. Wageningen university, Wageningen

Bentsink L, Koornneef M (2002) Seed dormancy and germination. In CR Somerville, EM Meyerowitz eds, The Arabidopsis book. The American Society of Plant Biologists, Rockville doi/10.1199/tab.0050, http://www.aspb.org/publications/arabidopsis

Bentsink L, Alonso-Blanco C, Vreugdenhil D, Tesnier K, Groot SPC, Koornneef M (2000) Genetic analysis of seed-soluble oligosacchrides in relation to seed storability of Arabidopsis. Plant Physiol 124: 1595-1604

Bernatzky R, Tanksley SD (1986) Genetics of actin-related sequences in tomato. Theor Appl Genet 72: 314-324

Bethke PC, Jones RL (2001) Cell death of barley aleurone protoplasts is mediated by reactive oxygen species. Plant J 25: 19-29

Bettey M, Finch-Savage WE, King GJ, Lynn JR (2000) Quantitative genetic analysis of seed vigour and pre-emergence seedling growth traits in *Brassica oleracea*. New Phytol 148: 227-286

Bettey M, Finch-Savage WE (1998) Stress protein content of mature *Brassica* seeds and their germination performance. Seed Sci Res 8: 347-355

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

Bewley JD, Black M (1994) Seeds: physiology of development and germination. Plenum Press, New York

Bies-Ethève N, da Silva Conceicao A, Giraudat J, Koornneef M, Léon-Kloosterziel KM, Valon C, Delseny M (1999) Importance of the B2 domain of the Arabidopsis ABI3 protein and 2S gene regulation. Plant Mol Biol 40: 1045-1054

Bleecker AB, Estelle MA, Somerville C, Kende H (1988) Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. Science 241: 1086-1089

Blokhina O, Virolainen E, Fagerstedt KV (2003) Antioxidants, oxidative damage and oxygen deprivation stress: a review. Ann Bot 91: 179-194

Bowman JL, Alvarez J, Weigel D, Meyerowitz EM, Smyth DR (1993) Control of flower development in *Arabidopsis thaliana* by *APETALA1* and interacting genes. Development 119: 721-743

Buitink J, Leprince O, Hemminga M, Hoekstra FA (2000) Molecular mobility in the cytoplasm: an approach to describe and predict lifespan of dry germplasm. Proc Natl Acad Sci USA 97: 2385-2390

Cakmak I, Strbac D, Marschner H (1993) Activities of hydrogen peroxide-scavenging enzymes in germinating wheat seeds. J Exp Bot 44: 127-132

Chang C, Kwok SF, Bleecker AB, Meyerowitz EM (1993) Arabidopsis ethylene-response gene *ETR1*: similarity of product to two-component regulators. Science 262: 539-544

Chao WS, Liu V, Thomson WW, Platt K, Walling LL (1995) The impact of chlorophyll-retention mutations, *d1d2* and *cyt-G1*, during embryogeny in soybean. Plant Physiol 107: 253-262

Chin HF (1995) Storage of recalcitrant seeds. In AS Basra ed, Seed Quality: basic mechanisms and agricultural implications. Food Product Press, pp 209-222

Clerkx EJM, Blankenstijn-de Vries H, Ruys GJ, Groot SPC, Koornneef M (2003) Characterization of *green seed*, an enhancer of *abi3-1* in Arabidopsis that affects seed longevity. Plant Physiol 132: 1077-1084

Cobbet CS, May MJ, Howden R, Rolls B (1998) The glutathione-deficient, *cadmium-sensitive* mutant *cad2-1* of *Arabidopsis thaliana* is deficient in γ-glutamylcysteine synthetase. Plant J 16: 73-78

Conklin PL, Norris SR, Wheeler GL, Williams EH, Smirnoff N, Last RL (1999) Genetic evidence for the role of GDP-mannose in plant ascorbic acid (vitamin C) biosynthesis. Proc Natl Acad Sci USA 96: 4198-4203

Conklin PL, Williams EH, Last RL (1996) Environmental stress sensitivity of an ascorbic acid deficient Arabidopsis mutant. Proc Natl Acad Sci USA 93: 9970-9974

Coolbear P (1995) Mechanisms of seed deterioration. In AS Basra ed, Seed Quality: basic mechanisms and agricultural implications. Food Product Press, pp 223-277

Corbineau F, Come D, (1993) The concept of dormancy in cereal seeds. Proceedings of the 4th International Workshop on Seeds, Basic and Applied Aspects of Seed Biology, Angers, France, July 20-24. pp 581-589

Cui KH, Peng SB, Xing YZ, Xu CG, Yu SB, Zhang Q (2002) Molecular dissection of seedling-vigour and associated physiological traits in rice. Theor Appl Genet 105: 745-753

De Paula M, Perez-Otaola M, Darder M, Torres M, Frutos G, Martinez-Honduvilla CJ (1996) Function of the ascorbate-glutathione cycle in aged sunflower seeds. Physiol Plant 96: 543-550

De Vos CHR, Kraak HL, Bino RJ (1994) Aging of tomato seeds involves glutathione oxidation. Physiol Plant 92: 131-139

Debeaujon I, Koorneef M (2000) Gibberellin requirement for Arabidopsis seed germination determined both by testa characteristics and embryonic abscisic acid. Plant Physiol 122: 415-424

Debeaujon I, Léon-Kloosterziel KM, Koornneef M (2000) Influence of the testa on seed dormancy, germination and longevity in Arabidopsis. Plant Physiol 122: 403-413

Dickson MH (1980) Genetic aspects of seed quality. Hort Sci 15: 771-774

Doerge RW (2002) Mapping and analysis of quantitative trait loci in experimental populations. Nature Rev Genet 3: 43-52

Eastmond PJ, Germain V, Lange PR, Bryce JH, Smith SM, Graham IA (2000) Postgerminative growth and lipid catabolism in oilseeds lacking the glyoxylate cycle. Proc Natl Acad Sci USA 97: 5669-5674

El-Assal SD, Alonso-Blanco C, Peeters AJM, Raz V, Koornneef M (2001) A QTL for flowering time in Arabidopsis reveals a novel allele of *CRY2*. Nature Genet 29: 435-440

Ellis RH, Hong TD, Roberts EH, Tao K-L (1990) Low moisture content limits to relations between seed longevity and moisture. Ann Bot 65: 493-504

Farnsworth E (2000) The ecology and physiology of viviparous and recalcitrant seeds. Ann Rev Ecol Syst 31: 107-138

Fath A, Bethke PC, Jones RL (2001) Enzymes that scavenge reactive oxygen species are down regulated prior to gibberellic acid induced programmed cell death in barley aleurone. Plant Physiol 126: 156-166

Feinbaum RL, Ausubel FM (1988) Transcriptional regulation of the Arabidopsis chalcone synthase gene. Mol Cell Biol 8: 1985-1992

Finkelstein R, Gampala SSL, Rock CD (2002) Abscisic acid signaling in seeds and seedlings. Plant Cell suppl 2002: S15-S45

Finkelstein R, Lynch TJ (2000) The Arabidopsis abscisic acid response gene *ABI5* encodes a basic leucine zipper transcription factor. Plant Cell 12: 599-609

Finkelstein R, (1994) Mutations at two new Arabidopsis ABA response lovi are similar to the *abi3* mutations. Plant J 5: 765-771

Foolad MR, Lin GY, Chen FQ (1999) Comparison of QTLs for seed germination under non-stress, cold stress and salt stress in tomato. Plant Breeding 118: 167-173

Frugoli JA, Hong Zhong H, Nuccio ML, McCourt P, McPeek MA, Thomas TL, McClung CR (1996) Catalase is encoded by a multigene family in *Arabidopsis thaliana* (L.) Heynh. Plant Physiol 112: 327-336

Gao W, Clancy JA, Han F, Prada D, Kleinhofs A, Ullrich SE (2003) Molecular dissection of a dormancy QTL region near the chromosome 7 (5H) L telomere in barley. Theor Appl Genet 107: 552-559

Garciarrubio A, Legaria JP, Covarrubias AA (1997) Abscisic acid inhibits germination of mature Arabidopsis seeds by limiting the availability of energy and nutrients. Planta 203: 182-187

Gazzani S, Gendall AR, Lister C, Dean C (2003) Analysis of the molecular basis of flowering time variation in accessions. Plant Physiol 132: 1107-1114

Gidrol X, Serghini H, Noubhani B, Mocouot B, Mazliak P (1989) Biochemical changes induced by accelerated ageing in sunflower seeds. I. Lipid peroxidation and membrane damage. Physiol Plant 79: 591-597

Giraudat J, Hauge BM, Valon C, Smalle J, Parcy F, Goodman HM (1992) Isolation of the Arabidopsis *ABI3* gene by positional cloning. Plant Cell 4: 1251-1261

Grace SC, Logan BA (2000) Energy dissipation and radical scavenging by plant phenylpropanoid pathway. Phil Trans R Soc Lond 355: 1499-1510

Greggains V, Finch-Savage WE, Quick WP, Atherton NM (2000) Putative desiccation tolerance mechanisms in orthodox and recalcitrant seeds of the genus *Acer*. Seed Sci Res 10: 317-327

Grene R (2002) Oxidative stress and acclimation mechanism in plants. In CR Somerville, EM Meyerowitz eds, The Arabidopsis book. The American Society of Plant Biologists, Rockville doi/10.1199/tab.0036, http://www.aspb.org/publications/arabidopsis/

Groot SPC, Soeda Y, Stoopen G, Konings MCJM, van der Geest AHM (2003) Gene expression during loss and regaining of stress tolerance at seed priming and drying. In: G Nicholás, KJ Bradford, D Côme, HW Pritchard eds: The biology of seeds, , ISBN 0851996531

Hampton JG, TeKrony DM (1995) Handbook of Vigour test methods, Ed 3. The international seed testing association, Zurich

Harada JJ (2001) Role of Arabidopsis *LEAFY COTYLEDON* genes in seed development. J. Plant Physiol 158: 405-409

Haslekas C, Stacy RAP, Nygaard V, Culianez MFA, Aalen RB (1998) The expression of a peroxiredoxin antioxidant gene, *AtPer1*, in *Arabidopsis thaliana* is seed specific and related to dormancy. Plant Mol Biol 36: 833-845

Hay FR, Mead A, Manger K, Wilson FJ (2003) One-step analysis of seed storage data and the longevity of *Arabidopsis thaliana* seeds. J Exp Bot 54: 993-1011

Hay FR, Probert RJ, Smith RD (1997) The effect of maturity on the moisture relations of seed longevity in foxglove (*Digitalis purpurea* L.). Seed Sci Res 7: 341-349

Heath RJ, Packer L (1968) Photoperoxidation in isolated chloroplasts. Arch Biochem Biophys 125: 189-198

Hendry GAF (1997) Free radicals in seeds- moving the debate forward. In RH Ellis, M Black, AJ Murdoch, TD Hong eds, Basic and applied aspects of seed biology. Kluwer academic publishers, Dordrecht, pp 657-663

Hendry GAF (1993) Oxygen, free radical processes and seed longevity. Seed Sci Res 3: 141-153

Hoekstra FA, Golovina EA, Buitink J (2001) Mechanisms of plant desiccation tolerance. Trends Plant Sci 6: 431-438

Hilhorst (1998) The regulation of secondary dormancy. The membrane hypothesis revisited. Seed Sci Res 8: 1-14

Hoffman MH (2002) Biogeography of *Arabidopsis thaliana*(L.) Heynh. (*Brassicaceae*). J Biogeography 29: 125-134

Holdsworth MJK, Swarup K, McKibbin, R (1999) Molecular and genetic mechanisms regulating the transition from embryo development to germination. Trends Plant Sci 4: 275-382

Hong S-W, Vierling E (2000) Mutants of Arabidopsis thaliana defective in acquisition to high temperature stress. Proc Natl Acad Sci 97: 4392-4397

Howden R, Andersen CR, Goldsbrough PB, Cobbet CS (1995) A cadmium sensitive, glutathion-deficient mutant of *Arabidopsis thaliana*. Plant Physiol 107: 1067-1073

Illipronti Jr. RA (1997) Variation in quality of individual seeds within a seed lot of soybean [*Glycine max* (L.) Merrill]. PhD thesis. Wageningen University, Wageningen

Ingram J, Bartels D (1996) The molecular basis of dehydration tolerance in plants. Annu Rev Plant Physiol Plant Mol Biol 47: 377-403

Izawa T, Takahashi Y, Yano M (2003) Comparative biology comes into bloom: genomic and genetic comparison of flowering pathways in rice and Arabidopsis. Curr Opinion Plant Biol 6: 113-120

Jalink H, van der Schoor R, Frandas A, van Pijlen JG, Bino RJ (1998) Chlorophyll fluorescence of *Brassica oleracea* seeds as a non-destructive marker for seed maturity and seed performance. Seed Sci Res 8: 437-443

Jander G, Norris SR, Rounsley SD, Bush DF, Levin IM, Last RL (2002) Arabidopsis map-based cloning in the post genome era. Plant Physiol 129: 440-450

Johnson-Flanagan AM, Maret LLD, Pomeroy MK (1994) Humidification of green canola seed leads to pigment degradation in the absence of germination. Crop Sci 34: 1618-1623

Johnson-Flanagan AM, Thiagarajah MR (1990) Degreening in canola (*Brassica napus*, cv, Westar) embryos under optimum conditions. J. Plant Physiol 136: 180-186

Kempin S, Savidge B, Yanofsky MF (1995) Molecular basis of the *cauliflower* phenotype in Arabidopsis. Science 267: 522-525

Khan MM, Hendry GAF, Atherton NM, Vertucci-Walters CW (1996) Free radical accumulation and lipid peroxidation in testas of rapidly aged soybean seeds: a light regulated process. Seed Sci Res 6: 101-107

Khurmatov KK (1982) Heterogeneity of natural populations of the *Arabidopsis thaliana* (Pamiro-Alay) in the flowering time. Arabid Inf Serv 19: 63-66

Kliebenstein D, Monde R, Last RL (1998) Superoxide dismutase in Arabidopsis: an eclectic enzyme family with disparate regulation and protein localization. Plant Physiol 118: 637-650

Koornneef M, Alonso-Blanco C, Bentsink L, Blankenstijn-de Vries H, Debeaujon I, Hanhart CJ, Leon-Kloosterziel KM, Peeters AJM, Raz V (2000) The genetics of seeds dormancy in *Arabidopsis thaliana*. In J-D Viémont, J Crabbé eds, Dormancy in plants. CABI publishing, pp 365-373

Koornneef M, Léon-Kloosterziel KM, Schwartz SH, Zeevaart JAD (1998) The genetic and molecular dissection of abscisic acid biosynthesis and signal transduction in Arabidopsis. Plant Physiol Biochem 36: 83-89

Koornneef M, Blankenstijn-de Vries H, Hanhart CJ, Soppe W, Peeters AJM (1994) The phenotype of some late-flowering mutants is enhanced by a locus on chromosome 5 that is not effective in the Landsberg *erecta* wild-type. Plant J 6: 911-919

Koornneef M, Hanhart CJ, Hilhorst HWM, Karssen CM (1989) In vivo inhibition of seed development and reserve protein accumulation in recombinants of abscisic acid biosynthesis and responsiveness mutants in *Arabidopsis thaliana*. Plant Physiol 90: 463-469

Koornneef M, Elgersma A, Hanhart CJ, Van Loenen-Martinet EP, van Rijn EP, Zeevaart JAD (1985) A gibberellin insensitive mutant of *Arabidopsis thaliana*. Physiol Plant 65: 33-39

Koornneef M, Hanhart CJ (1984) The localization of two *ABA-insensitive* genes. Arabid Inf Serv 21: 5-9

Koornneef M, Van der Veen JH (1980) Induction and analysis of gibberellin-sensitive mutants in *Arabidopsis thaliana* (L.) Heynh. Theor Appl Genet 58: 257-263

Kulik MM (1995) Seed quality and microorganisms. In AS Basra ed, Seed Quality: basic mechanisms and agricultural implications. Food Product Press, pp 173-207

Kwong FY (1991) Research needs in the production of high quality seeds. In J Prakash, RLM Pierik eds, Horticulture- New technologies and applications. Kluwer Academic Publishers, Dordrecht, pp 13-20

Kwong F, Bui AQ, Lee H, Kwong LW, Fisher RL, Goldberg RB, Harada JJ (2003) *Leafy cotelydon1-like* defines a class of regulators essential for embryo development. Plant Cell 15: 5-18

Larkindale J, Knight MR (2002) Protection against heat stress-induced oxidative damage in Arabidopsis involves calcium, abscisic acid, ethylene and salycylic acid. Plant Physiol 128: 682-695

Lee B-H, Lee H, Xiong L, Zhu J-K (2002) A mitochondrial complex I defect impairs cold-regulated nuclear gene expression. Plant Cell 14: 1235-1251

Léon-Kloosterziel KM, van de Bunt GA, Zeevaart JAD, Koornneef M (1996a) Arabidopsis mutants with a reduced seed dormancy. Plant Physiol 110: 233-240

Léon-Kloosterziel KM, Alvarez-Gil M, Ruys GJ, Jacobsen SE, Olszewski NE, Schwartz SH, Zeevaart JAD, Koornneef M (1996b) Isolation and characterization of *abscisic acid-deficient* Arabidopsis mutants at two new loci. Plant J 10: 655-661

Léon-Kloosterziel KM, Keijzer CJ, Koornneef M (1994) A seed shape mutant of Arabidopsis that is affected in integument development. Plant Cell 6: 385-392

Leprince O, Hoekstra FA (1998) The response of cytochrome redox state and energy metabolism to dehydration support a role for cytoplasmic viscosity in desiccation tolerance. Plant Physiol 118: 1253-1264

Leprince O, Atherton NM, Deltour R, Hendry GAF (1994) The involvement of respiration in free radical processes during loss of desiccation tolerance in germinating *Zea mays* L. Plant Physiol 104: 1333-1339

Leprince O, Hendry GAF, McKersie BD (1993) The mechanisms of desiccation tolerance in developing seeds. Seed Sci Res 3: 231-246

Leung J, Giraudat J (1998) Abscisic acid signal transduction. Annu Rev Plant Physiol Plant Mol Biol 49: 199-222

Lindstrom EW (1942) Inheritance of seed longevity in maize hybrids. Genetics 27: 154

Lister C, Dean C (1993) Recombinant inbred lines for mapping RFLP and phenotypic markers in *Arabidopsis thaliana*. Plant J. 4: 745-750

Lotan T, Ohto M, Yee KM, West MAL, lo R, Kwong RW, Yamagishi K, Fischer RL, Goldberg RB, Harada JJ (1998) Arabidopsis *LEAFY COTYLEDON1* is sufficient to induce embryo development in vegetative cells. Cell 93: 1195-1205

Loudet O, Chaillou S, Camilleri C, Bouchez D, Daniel-Vedele F (2002) Bay-0 X Shahdara recombinant inbred line population: a powerful tool for genetic dissection of complex traits in Arabidopsis. Theor Appl Genet 104: 1173-1184

Lu Y-P, Li Z-S, Drozdowicz YM, Hörtgensteiner S, Martinoia E, Rea PA (1998) *AtMRP2*, an Arabidopsis ATP binding cassette transporter able to transport glutathione S-Conjugates and chlorophyll catabolites: functional comparisons with *AtMRP1*. Plant Cell 10: 267-282

Mano Y, Takeda K (1997) Mapping quantitative trait loci for salt tolerance at germination and the seedling stage in barley (*Hordeum vulgare* L.). Euphytica 94: 263-272

Matin A, Nadeau JA (2001) Sensitized polygenetic trait analysis. Trends Genet 17: 727-731

McDonald MB (1999) Seed deterioration: Physiology, repair and assessment. Seed Sci Technol 27: 177-237

McDonald MB (1998) Seed quality assessment. Seed Sci Res 8: 265-275

McGee DC (2000) Pathology of seed deterioration. In: SH Moore, RW Yaklich eds Genetic improvement of seed quality, Ed 31, Vol XVII. Madison, Wisconsin: Crop Science Society of America, Anaheim, pp 21-37

McKay JK, Richards JH, Mitchell-Olds T (2003) Genetics of drought adaptation in *Arabidopsis thaliana*: I. pleiotophy contributes to genetic correlations among ecological traits. Mol Ecol 12: 1137-1151

Meinke DW, Meinke LK, Showalter TC, Shissel AM, Mueller LA, Tzafrir I (2003) A sequence-based map of Arabidopsis genes with mutant phenotypes. Plant Physiol 131: 409-418

Meinke DW, Cherry JM, Dean C, Rounsley SD, Koornneef M (1998) *Arabidopsis thaliana*: A model plant for genome analysis. Science 282: 662-682

Meinke DW, Franzmann LH, Nickle TC, Yeung EC (1994) *Leafy cotyledon* mutants of Arabidopsis. Plant Cell 6: 1049-1064

Meinke DW (1992) A homeotic mutant of *Arabidopsis thaliana* with leafy cotyledons. Science 258: 1647-1650

Meyer K, Leube MP, Grill E (1994) A protein phosphatase 2C involved in ABA signal transduction in *Arabidopsis thaliana*. Science 264: 1452-1455

Michelmore RW, Paran I, Kesseli RV (1991) Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. Proc Natl Acad Sci USA 88: 9828-9832

Miura K, Lin SY, Yano M, Nagamine T (2002) Mapping quantitative trait loci controlling seed longevity in rice (*Oryza sativa* L.). Theor Appl Genet 104: 981-986

Moore SH, Halloin JM (2000) Genetic resistance to seed deterioration. In: SH Moore, RW Yaklich eds genetic improvement of seed quality, Ed 31, Vol XVII. Madison, Wisconsin: Crop Science Society of America, Anaheim, pp 21-37

Nadeau JA (2001) Modifier genes in mice and humans. Nature Rev Genet 2: 165-174

Nambara E, Suzuki M, Abrams S, McCarty DR, Kamiya Y, McCourt P (2002) A screen for genes that function in abscisic acid signaling in *Arabidopsis thaliana*. Genetics 161: 1247-1255

Nambara E, Keith K, McCourt P, Naito S (1995) A regulatory role for the *ABI3* gene in the establishment of embryo maturation in *Arabidopsis thaliana*. Development 121: 629-636

Nambara E, Naito S, McCourt P (1992) A mutant of Arabidopsis which is defective in seed development and storage protein accumulation is a new *abi3* allele. Plant J 2: 435-441

Natoli A, Gorni C, Chegdani F, Ajmone Marsan P, Colombi C, Lorenzoni C, Marocco A (2002) Identification of QTLs associated with sweet sorghum quality. Maydica 47: 311-322

Neff MM, Neff JD, Chory J, Pepper AE (1998) dCAPS, a simple technique for the genetic analysis of single nucleotide polymorphisms: experimental applications in *Arabidopsis thaliana* genetics. Plant J 14: 387-392

Nocter G, Foyer CH (1998) Ascorbate and glutathion:keeping active oxygen under control. Ann Rev Plant Physiol Plant Mol Biol 49: 249-279

Obendorf RL (1997) Oligosaccharides and galactosyl cyclitols in seed desiccation tolerance. Seed Sci Res 7: 63-74

Oliver AE, Leprince O, Wolkers WF, Hincha DK, Heyer AG, Crowe JH (2001) Non-disaccharide-based mechanisms of protection during drying. Cryobiology 43: 151-167

Ooms JJJ, Léon-Kloosterziel KM, Bartels D, Koornneef M, Karssen CM (1993) Acquisition of desiccation tolerance and longevity in seeds of *Arabidopsis thaliana* - A comparative study using ABA insensitive *abi3* mutants. Plant Physiol 102: 1185-1191

Overmyer K, Brosche M, Kangasjarvi J (2003) Reactive oxygen species and hormonal control of cell death. Trends Plant Sci 8: 335-342

Page DR, Grossniklaus U (2002) The art and design of genetic screens: *Arabidopsis thaliana*. Nature Rev Genet 3: 124-136

Pandey DK (1992) Conductivity testing of seeds. In HF Linskens, JF Jackson, LA Amberger, JC Autran, D Bullock eds, Seed analysis, Vol 14. Springer, Berlin, pp 273-304

Parcy F, Valon C, Kohara A, Miséra S, Giraudat J (1997) The *ABSCISIC ACID-INSENSITIVE3*, *FUSCA3*, and *LEAFY COTOLEDON1* loci act in concert to control multiple aspects of Arabidopsis seed development. Plant Cell 9: 1265-1277

Parcy F, Valon C, Raynal M, Gaubier-Comella P, Delseny M, Giraudat J (1994) Regulation of gene expression programs during Arabidopsis seed development: roles of the *ABI3* locus and of endogenous abscisic acid. Plant Cell 6: 1567-1582

Peeters AJM, Blankestijn-de Vries H, Hanhart, CJ, Léon-Kloosterziel, KM, Zeevaart JAD, Koornneef M (2002) Characterization of mutants with reduced seed dormancy at two novel rdo loci and a further characterization of *rdo1* and *rdo2* in Arabidopsis. Physiol Plant 115: 604-612

Peng J, Carol P, Richards DE, King KE, Cowling RJ, Murphy GP, Harberd NP (1997) The Arabidopsis *GAI* gene defines a signaling pathway that negatively regulates gibberellin responses. Genes and Development 11: 3194-3205

Pill WG (1995) Low water potential and pre-sowing germination treatments to improve seed quality. In AS Basra ed, Seed Quality: basic mechanisms and agricultural implications. Food Product Press, pp 319-359

Price J, Li T-C, Kang G, Na JK, Jang J-C (2003) Mechanisms of glucose signaling during germination of Arabidopsis. Plant Physiol 132: 1424-1438

Pritchard SL, Charlton WL, Baker A, Graham IA (2002) Germination and storage reserve mobilization are regulated independently in Arabidopsis. Plant J 31: 639-647

Puntarulo S, Galleano M, Sanchez RA, Boveris A (1991) Superoxide anion and hydrogen peroxide metabolism in soybean embryonic axes during germination. Biochim Biophys Acta Int J Biochem Biophys 1074: 277-283

Quesada A, Garcia-Martinez S, Piqueras P, Ponce MR, Micol JL (2002) Genetic architecture of NaCl tolerance in Arabidopsis. Plant Physiol 130: 951-963

Quesada V, Ponce MR, Micol JL (2000). Genetic analysis of salt-tolerant mutants in Arabidopsis thaliana. Genetics 154:421-36.

Raz V, Bergervoet JHW, Koornneef M (2001) Sequential steps for developmental arrest in Arabidopsis seeds. Development 128: 243-252

Rice-Evans CA, Miller NJ, Paganga G (1997) Antioxidant properties of phenolic compounds. Trends Plant Sci 2: 152-159

Rock CD, Zeevaart JAD (1991) The *aba* mutant of *Arabidopsis thaliana* is impaired in epoxy-carotenoid biosynthesis. Proc Natl Acad Sci USA 88: 7496-7499

Salomé PA, McClung CR (2002) Characterization of the Arabidopsis catalase family XIII International Conference on Arabidopsis Research, Sevilla, Spain

Schopfer P, Plachy C, Frahry G (2001) Release of reactive oxygen intermediates (superoxide radicals, hydrogen peroxide, and hydroxyl radicals) and peroxidase in germinating radish seeds controlled by light, gibberellin, and abscisic acid. Plant Physiol 125: 1591-1602

Senaratna T, Gusse JF, McKersie BD (1988) Age-induced changes in cellular membranes of imbibed soybean seed axes. Physiol Plant 73: 85-91

Shen Z-X, Parrish DJ, Wolf DD, Welbaum GE (2001) Stratification of switchgrass seeds is reversed and hastened by drying. Crop Sci 41: 1546-1551

Shirley BW, Kubasek WL, Storz G, Bruggemann E, Koornneef M, Ausubel FM, Goodman HM (1995) Analysis of Arabidopsis mutants deficient in flavonoid biosynthesis. Plant J 8: 659-671

Sinniah UR, Ellis RH, John P (1998) Irrigation and seed quality development in rapid-cycling *Brassica*; seed germination and longevity. Ann Bot 82: 309-314

Stacy RAP, Nordeng TW, Culianez MFA, Aalen RB (1999) The dormancy related peroxiredoxin antioxidant, *PER 1*, is localized to the nucleus of barley embryo and aleurone cells. Plant J 19: 1-8

Staswick PE, Tiryaki I, Rowe ML (2002) Jasmonate response locus *JAR1* and several related Arabidopsis genes encode enzymes of the firefly luciferase superfamily that show activity on jasmonic, salicylic, and indole-3-acetic acids in an assay for adenylation. Plant Cell 14: 1405-1415

Staswick PE, Su W, Howell SH (1992) Methyl jasmonate inhibition of root growth and induction of leaf protein are decreased on an *Arabidopsis thaliana* mutant. Proc Natl Acad Sci USA 89: 6837-6840

Steber CM, Cooney SE, McCourt P (1998) Isolation of the GA-response mutant *sly1* as a suppressor of *ABI1-1* in *Arabidopsis thaliana*. Genetics 149: 509-521

Steckel JRA, Gray D, Rowse HR (1989) Relationships between indices of seed maturity and carrot seed quality. Ann Appl Biol 114: 177-183

Sun T, Goodman HM, Ausubel FM (1992) Cloning the Arabidopsis *GA1* locus by genomic subtraction. Plant Cell 4: 119-128

Swarup K, Alonso-Blanco C, Lynn JR, Michaels SD, Amasino RM, Koornneef M, Millar AJ (1999) Natural allelic variation identifies new genes in the Arabidopsis circadian system. Plant J 20: 67-77

Taji T, Seki M, Yamaguchi Shinozaki K, Kamada H, Giraudat J, Shinozaki K (1999) Mapping of 25 drought-inducible genes. *RD* and *ERD*, in *Arabidopsis thaliana*. Plant Cell Physiol 40: 119-123

Tesnier K, Strookman-Donkers HM, van Pijlen JG, van der Geest AHM, Bino RJ, Groot SPC (2002) A controlled deterioration test of *Arabidopsis thaliana* reveals genetic variation in seed quality. Seed Sci Technol 30: 149-165

Thomas H, Ougham H, Canter P, Donnison I (2002) What stay-green mutants tell us about nitrogen remobilization in leaf senescence. J Exp Bot 53: 801-808

Thomas H, Schellenberg M, Vicentini F, Matile P (1996) Gregor Mendel's green and yellow pea seeds. Botanica Aata 109: 3-4

Thomas H, Smart CM (1993) Crops that stay green. Ann Appl Biol 123: 193-219

Thorlby G, Veale E, Butcher K, Warren G (1999) Map positions of *SFR* genes in relation to other freezing related genes in *Arabidopsis thaliana.* Plant J 17:445-52

Tommasi F, Paciolla C, Concetta de Pinto M, Da Gara L (2001) A comparative study of glutathione and ascorbate metabolism during germination of *Pinus pinea* L. seeds. J Exp Bot 52: 1647-1654

Tommasi F, Paciolla C, Arrigoni O (1999) The ascorbate system in recalcitrant and orthodox seeds. Physiol Plant 105: 193-198

Tsuchiya T, Ohta H, Okawa K, Iwamatsu A, Shimada H, Masuda T, Takamiya K-I (1999) Cloning chlorophyllase, the key enzyme in chlorophyll degradation: Finding of a lipase motif and the induction by methyl jasmonate. Proc Natl Acad Sci USA 96: 15362-15367

Van der Schaar W, Alonso-Blanco C, Léon-Kloosterziel KM, Jansen RC, Van Ooijen JW, Koornneef M (1997) QTL analysis of seed dormancy in Arabidopsis using recombinant inbred lines and MQM mapping. Heredity 79: 190-200

Van Ooijen JW, Maliepaard C (1996) MapQTL™ version 4.0: software for the calculation of QTL positions on genetic maps. Plant research International, Wageningen, The Netherlands.

Van Ooijen JW (1992) Accuracy of mapping quantitative trait loci in autogamous species. Theor Appl Genet 84: 803-811

Ward K, Scarth R, Daun JK, Vessey JK (1995) Chlorophyll degradation in summer oilseed rape and summer turnip during seed ripening. Can J Plant Sci 75: 413-420

Wehmeyer N, Vierling E (2000) The expression of small heat shock proteins in seeds responds to discrete developmental signals and suggests a general protective role in desiccation tolerance. Plant Physiol 122: 1099-1108

Wesley-Smith J, Walters C, Pammenter NW, Berjak P (2001) Interactions among water content, rapid (non-equilibrium) cooling to -196°C, and survival of embryonic axes of *Aesculus hippocastaneum* L. seeds. Cryobiology 42: 196-206

West MAL, Matsudaira Yee K, Danao J, Zimmerman JL, Fisher RL, Goldberg RB, Harada JJ (1994) *LEAFY COTYLEDON1* is an essential regulator of late embryogenesis and cotyledon identity in Arabidopsis. Plant Cell 6: 1731-1745

Willekens H, Inzé D, Montagu van M, van Camp W (1995) Catalases in plants. Molec Breeding 1: 207-228

Wilson Jr. DO (1995) The storage of orthodox seeds. In AS Basra ed, Seed Quality: basic mechanisms and agricultural implications. Food Product Press, pp 173-208

Wilson IW, Schiff CL, Hughes DE, Somerville SC (2001) Quantitative trait loci analysis of powdery mildew disease resistance in the Arabidopsis thaliana accessions Kashmir-1. Genetics 158: 1301-1309

Wolkers WF, Alberda M, Koornneef M, Léon-Kloosterziel KM, Hoekstra FA (1998) Properties of proteins and the glassy matrix in maturation defective mutant seeds of *Arabidopsis thaliana*. Plant J 16: 133-143

Wüthrich KL, Bovet L, Hunziker PE, Donnison I, Hörtgensteiner S (2000) Molecular cloning, functional expression and characterization of RCC reductase involved in chlorophyll catabolism. Plant J 21: 189-198

Xiong L, Schumaker KS, Zhu J-K (2002) Cell signaling during cold, drought, and salt stress. Plant Cell 14 supplement: 165-183

Xiong L, Zhu J-K (09/30/2002) Salt tolerance. In CR Somerville, EM Meyerowitz eds, The Arabidopsis Book. The American Society of Plant Biologists, Rockville doi/10.1199/tab.0048, http://www.aspb.org/publications/arabidopsis/

Samenvatting

Overleven in ongunstige groeiperiodes, bijvoorbeeld in de winter of tijdens een droge zomer, is voor planten van essentieel belang. Overleven als rijp zaad is een van de mogelijkheden die planten hiertoe hebben ontwikkeld. In de natuur komen twee typen zaden voor: “natte”, zoals de kastanje, en “droge” zoals granen, erwten enz. Sommige zaden van de “droge” groep, die we orthodoxe zaden noemen, kunnen soms na 200 jaar bewaring nog kiemen. Er zijn voorspellingen dat sommige zaden, opgeslagen onder de juiste omstandigheden, tot wel 2000 jaar bewaard kunnen worden zonder hun kiemkracht te verliezen.

In dit proefschrift is onderzoek beschreven naar de erfelijke factoren (genen) die van invloed zijn op de zaadkwaliteit, waarbij het vermogen van zaden om bewaring te overleven een belangrijke eigenschap is.

Zaden van hoge kwaliteit kiemen steeds goed en leveren sterke kiemplanten, ook na bewaring. De kwaliteit van zaden wordt beïnvloed door de gezondheid van de moederplant en die is afhankelijk van bijvoorbeeld weersomstandigheden en eventuele ziektes. Daarnaast spelen de omstandigheden waaronder de geoogste zaden opgeslagen worden een belangrijke rol. Behandelingen zoals “priming”, die toegepast worden om de kieming van partijen zaad te verbeteren, beïnvloeden de bewaarbaarheid van zaden ook. Onderzoek heeft uitgewezen dat orthodoxe zaden, die onder lage relatieve luchtvochtigheid en temperatuur opgeslagen worden jaren goed blijven. Toch kunnen twee rassen van hetzelfde gewas, opgekweekt en bewaard onder dezelfde omstandigheden sterk verschillen in zaadkwaliteit. Dit wordt veroorzaakt door erfelijke verschillen. Over de genen die deze kwaliteitsverschillen bepalen, weten we nog maar heel weinig. Kennis van de genen die van invloed zijn op de bewaring van zaden kan in plantenveredelingsprogramma’s gebruikt worden om rassen voor deze eigenschap te verbeteren, vergelijkbaar met het selecteren op andere eigenschappen zoals opbrengst en kwaliteit (bijvoorbeeld smaak van tomaten).

In dit onderzoek is gebruik gemaakt van de modelplant: *Arabidopsis thaliana* (zandraket). Deze plant heeft als voordeel dat er veel genetische variatie beschikbaar is, die gebruikt kan worden om de functie van genen te ontrafelen. Genetische variatie betekent dat er erfelijke verschillen zijn tussen rassen van deze soort, net zoals er veel verschillende tomaten -, bieten - en tarwerassen zijn. Deze variatie kan door de mens opzettelijk gemaakt zijn door mutagenese, maar is ook aanwezig in de natuur. Mutagenese is een kunstmatige methode om variatie te maken. Hierbij worden willekeurig de genen beschadigd, waarna men de voor het onderzoek interessante mutant moet uitzoeken (selecteren) uit een grote hoeveelheid planten waarin mutaties voorkomen. In genetische studies wordt vaak gebruik gemaakt van mutanten waarin één enkel gen defect is. Bij Arabidopsis kunnen ongeveer 5

tot 6 generaties per jaar gekweekt worden en dat versnelt het erfelijkheidsonderzoek. Ook komt er steeds meer bewijs dat de functie van genen bij verschillende plantensoorten geconserveerd is, hetgeen betekent dat als je de functie van een gen in een soort weet, je op zoek kunt gaan naar eenzelfde gen in een andere soort.

In hoofdstuk 2 is gekeken naar de rol die anti-oxidanten en de zaadhuid spelen bij het optreden van schade tijdens bewaring van zaden. Anti-oxidanten die hier bestudeerd zijn, zijn vitamine-C, gluthation, flavenoiden en katalase. Er was de beschikking over mutanten die een lagere hoeveelheid van deze stoffen bevatten. Anti-oxidanten neutraliseren het effect van vrije radicalen. Vrije radicalen kunnen op verschillende manieren ontstaan en kunnen celcomponenten beschadigen. De zaadhuid werd bestudeerd door gebruik te maken van mutanten die een afwijkende zaadhuid hebben. Omdat het mogelijk is dat een defect in één enkel gen een gering of geen effect zou hebben, hebben we deze defecten ook gecombineerd in dubbelmutanten. Het effect van al deze mutaties werd bestudeerd op twee manieren. Ten eerste met een CD-toets en ten tweede door zaden te laten kiemen in aanwezigheid van vrije radicalen. Een CD-toets, “controlled deterioration”, versneld het verouderingsproces van zaden door ze een aantal dagen bloot te stellen aan een hoge relatieve luchtvochtigheid (= RV 80%) en temperatuur (40°C). We kunnen dan sneller het effect op bewaring bepalen van bijvoorbeeld een verlaging in de hoeveelheid vitamine-C. We konden in beide testen weinig effect zien van een verminderde hoeveelheid van de hier bestudeerde anti-oxidanten. Hiervoor zijn verschillende mogelijke verklaringen; één ervan is dat andere anti-oxidanten zoals vitamine-E, peroxiredoxine en superoxide dismutase een belangrijkere rol spelen dan de hier onderzochte anti-oxidanten. De zaden met een afwijkende zaadhuid waren in beide toetsen wel gevoelig voor “bewaringsschade” en dit bewijst de noodzaak van een “functionele” zaadhuid voor bewaring.

Hoofdstuk 3 beschrijft een dubbelmutant die groenzadig (*green seed* = *grs*) is. In de literatuur was beschreven dat groene zaden slechter bewaarbaar waren dan niet groene zaden. Wij hebben deze hypothese getoetst met deze nieuwe groen zadige mutant. We konden inderdaad aantonen dat de *grs* zaden sneller doodgingen in een CD-toets. De zaden zijn ook opgeslagen bij een hogere relatieve luchtvochtigheid (= RV 60%) en constante temperatuur (20°C). In deze bewaringsproef kwam ook naar voren dat de *grs* zaden slechter bewaarbaar waren. Daarnaast bleek dat de *grs* zaden meer van hun celinhoud verliezen als ze enige tijd in water gelegd worden. Hieruit kan als voorzichtige conclusie getrokken worden dat de membranen, die de celinhoud op zijn plaats moeten houden, niet zo goed meer functioneren als in gezonde zaden.

Het effect van mutaties kan naar twee kanten werken. Ze kunnen zaden slechter maken zoals hierboven beschreven is, ze kunnen zaden ook beter bewaarbaar maken. Bij de selectie op bewaarbaarheid doet zich het probleem voor dat het lang duurt voordat je de genetische verschillen kunt waarnemen wanneer de zaden op normale wijze bewaard worden. Door echter gebruik te maken van een bestaande mutant, waarvan de zaden maar 6 tot 8 weken levend blijven, konden we in deze genetische achtergrond erfelijke verschillen waarnemen, binnen een redelijke tijd, in zaden die op normale manier bewaard waren. De *abi3-5* mutant is onder normale omstandigheden maar beperkt houdbaar, ongeveer 3 maanden. In een mutagenese-experiment, uitgaande van deze *abi3-5* mutant is geselecteerd op zaden die langer dan deze tijd bewaarbaar zijn. Planten opgekweekt uit deze zaden die veel langer dan 3 maanden bewaarbaar zijn, hebben dan een mutatie in een gen dat bewaarbaarheid van zaden beïnvloedt. In hoofdstuk 4 is de selectie van vier nieuwe mutanten beschreven die de potentie bezitten om de bewaarbaarheid te verbeteren.

In hetzelfde hoofdstuk is ook beschreven hoe gebruik gemaakt kon worden van de variatie die in de natuur te vinden is. Arabidopsis kun je in grote delen van de wereld tegen komen en deze Arabidopsisrassen uit verschillende plaatsen en landen verschillen voor veel eigenschappen wel iets van elkaar. Er is weer gebruik gemaakt van de planten die zaden dragen die maar beperkt bewaarbaar waren: de *abi3-5* mutant die ongeveer 3 maanden bewaarbaar is en de *lec1-3* mutant waarvan de zaden na 6 tot 8 weken al dood zijn. Deze mutanten zijn gekruist met 20 arabidopsisrassen uit de hele wereld en in de nakomelingschap van deze kruisingen is er geselecteerd op planten die zaden opleverden die langer bewaarbaar waren, terwijl ze nog wel de *abi3-5* of *lec1-3* mutatie bevatten. Deze selecties bevatten specifieke genen die de bewaarbaarheid van *abi3* en *lec1-3* mutante zaden beïnvloeden. De betreffende genen worden modificerende genen genoemd, omdat ze de eigenschap bewaarbaarheid beïnvloeden.

In hoofdstuk 5 is natuurlijke variatie op een andere manier gebruikt om genen te vinden die van invloed zijn op zaadkwaliteit. We hebben gebruik gemaakt van zogenaamde “Recombinant Inbred Lines = RILs” en een genetische techniek die “Quantitative Trait Mapping” heet. RILs zijn homozygote (zaadvaste) lijnen die ontstaan zijn door eerst twee rassen met elkaar te kruisen. Daarna is van de hybride die uit deze kruising afkomstig was zaad geoogst dat vervolgens door steeds weer zelfbevruchting (intelen) toe te passen homozygoot gemaakt is. In totaal is er gebruik gemaakt van 114 van die ingeteelde lijnen, die we RILs noemen. De QTL-analyse wordt uitgevoerd met daarvoor ontwikkelde computerprogramma’s, die dan een waarschijnlijke plaats berekenen voor een gen dat een rol speelt in de bestudeerde eigenschap. Zo’n waarschijnlijke plaats noemen we dan een “quantitative trait locus” (QTL). Eén ouder, Shakdara (Sha), gebruikt voor de oorspronkelijke

kruising, komt uit Tadjikistan in Centraal-Azië en de andere ouder, Landsberg *erecta* (L*er*), komt uit Polen. Aan al die 114 RILs zijn een aantal metingen gedaan. Zo is er gekeken naar hoe goed de zaden een CD-toets overleven, hoe goed ze kiemen op een oxidant en hoe zaden een hoge temperatuurbehandeling overleven, voordat je ze laat kiemen. Daarnaast hebben we ze te kiemen gezet op een hoge concentratie zout en mannitol en we hebben de eigenschap kiemrust bestudeerd. Voor de verschillende eigenschappen zijn QTLs gevonden. In sommige gevallen bleken QTLs voor verschillende eigenschappen op ongeveer dezelfde plaats te liggen. Dit zou kunnen betekenen dat eenzelfde gen twee van die eigenschappen beïnvloedt wat kan betekenen dat er eenzelfde mechanisme ten grondslag ligt aan bijvoorbeeld de gevoeligheid voor mannitol en zout tijdens kieming. Uit die QTL-posities bleek ook, dat er maar een beperkt aantal plaatsen is waar genen liggen die het kiemgedrag in al de hierboven genoemde omstandigheden beïnvloeden.

Het in dit proefschrift beschreven onderzoek heeft laten zien, dat het mogelijk is om op verschillende manieren aan te tonen dat er genen zijn die zaadkwaliteit beïnvloeden.

In hoofdstuk 6 hebben we al die mogelijke locaties van genen in een genetische kaart geplaatst. Deze kaart is tezamen met de verkregen mutanten en zogenaamde ‘modifier’-lijnen een belangrijk uitgangspunt voor het moleculair karakteriseren van deze genen. Door deze karakterisering kan hun werking mogelijk begrepen worden en ook gebruikt worden om de veredeling op zaadkwaliteit mogelijk te maken.

Publications

Clerkx EJM, Blankestijn – De Vries H, Ruys GJ, Groot SPC, Koornneef M (2003) Characterization of *green seed*, an enhancer of *abi3-1* in Arabidopsis that affects seed longevity. Plant Physiol 132: 1077-1084

Submitted for publication

Clerkx EJM, Blankestijn-De Vries H, Ruys GJ, Groot SPC, Koornneef M
Genetic differences in seed longevity of various Arabidopsis mutants

Clerkx EJM, El-Lithy ME, Vierling E, Ruys GJ , Blankestijn-De Vries H, Groot SPC, Vreugdenhil D, Koornneef M
Analysis of natural allelic variation of Arabidopsis seed quality traits between the accessions Landsberg *erecta* and Shakdara, using a new recombinant inbred line population

El-Lithy ME, Clerkx EJM, Ruys GJ, Koornneef M, Vreugdenhil D
QTL analysis of growth related traits in a new Arabidopsis recombinant inbred population

Curriculum vitae

Emile Clerkx werd op 24 mei 1971 geboren te Geleen. Na het behalen van het VWO-diploma in 1989 aan de Albert Schweitzer Scholengemeenschap (tegenwoordig Graaf Huyn College) in Geleen, begon hij in hetzelfde jaar met de studie Plantenveredeling aan de Landbouwuniversiteit te Wageningen (tegenwoordig Wageningen Universiteit). Tijdens de doctoraalfase werden de afstudeervakken Erfelijkheidsleer en Plantenveredeling gevolgd. De stageperiode werd vervuld bij de Commonwealth Scientific and Industrial Research Organization te Canberra, Australië. Na zijn afstuderen was hij onder andere werkzaam bij het Agrotechnologisch Onderzoeksinstituut (tegenwoordig Agrotechnology & Food Innovations) te Wageningen. In januari 1999 begon hij met een promotieonderzoek bij de leerstoelgroep voor Erfelijkheidsleer van Wageningen Universiteit. Tijdens deze periode deed hij onderzoek naar de genetische regulatie van zaadkwaliteit. De resultaten van dit onderzoek, onder begeleiding van Prof. dr. ir. Maarten Koornneef en dr. Steven P.C. Groot, staan beschreven in dit proefschrift.

Nawoord

A long time ago in a galaxy far, far away…….
Zo begint elk deel van een filmcyclus die mij al boeit sinds het eerste deel verscheen in 1977: Star Wars. Het klassieke verhaal van de goeden die tegen de slechten vechten, gesitueerd in de ruimte. Ik moet toegeven dat ik geen slechte mensen ben tegengekomen tijdens mijn promotieonderzoek, wel heel veel goede. Die goede wil ik graag bedanken voor al hun hulp en als ik mezelf dan neerzet als Luke Skywalker, hoewel ik lang niet zo heldhaftig heb hoeven vechten als hij, kan ik proberen de rest te vergelijken met de helden uit de films. Beginnen doe ik uiteraad met Yoda, de grote leermeester, die mij eind 1998 dolblij maakte door me te accepteren als zijn leerling. Maarten, jou fysiek vergelijken met Yoda zou een belediging zijn, maar in wijsheid en bevlogenheid voor datgene wat je mensen kunt en wilt leren ben je zeker zijn evenknie. Luke wilde “Jedi knight” worden. Ik had na mijn afstuderen één wens en dat was AIO worden en als ik dan een leermeester mocht kiezen dan het liefst Yoda, de grote leermeester. Wensen gaan niet altijd in vervulling, de mijne toen wel. Daarom bedankt voor alles wat je tijdens mijn opleiding tot “Jedi knight” voor mij betekend hebt. Yoda kan niet alles alleen overzien, vandaar dat hij in het veld de hulp inroept van Obi-Wan; die rol wil ik toebedelen aan mijn co-promotor Steven. Steven, en alle mensen die op PRI mijn werk mogelijk hebben gemaakt, hartelijk bedankt voor het beschikbaar stellen van de faciliteiten en de praktische en theoretische kennis die je met mij hebt willen delen. Luke kan niet zonder hulp van zijn trouwe robots, R2D2 en C3PO. Die rol deel ik toe aan Hetty en Gerda. Het is misschien niet leuk om vergeleken te worden met robots, ze zijn echter precies, klagen nooit over de hoeveelheid werk en routinematig werk wordt zonder morren uitgevoerd, eigenschappen waar ik niet zonder zou hebben gekund. Hetty en Gerda, zonder jullie hulp zou ik nooit de hoeveelheid plantselecties, kiemproeven, DNA-isolaties en CD-testen hebben kunnen doen, die nodig waren voor de totstandkoming van dit proefschrift. Met de werkzaamheden die ik genoemd heb, doe ik jullie nog tekort; jullie waren voor mij dus even onmisbaar als R2D2 en C3PO waren voor de goede afloop van Luke’s avonturen. Ik wil bij dezen ook Corrie bedanken, die regelmatig heeft meegeholpen met proeven die weer eens wat groter in omvang waren dan gepland. Ik hoop dat je het niet erg vindt als er even geen belangrijke robot meer voorhanden is. Je hulp wordt er zeker niet minder om gewaardeerd. Uiteraard horen daar ook de gesprekken bij die gevoerd werden als we tijdens de pauzes verbannen waren naar het rokersgedeelte. Belangrijke feedback wordt in de film verkregen van de “Jedi-council”: het adviesorgaan. Mijn “Jedi-council” bestond uit de begeleidingscommisie van STW en Dick, Steve en Elizabeth. Ik weet van de mensen uit de begeleidingscommisie dat er op bedrijven weinig problemen zijn met langdurige bewaring van zaden, maar ik hoop dat jullie de kennis daarover, verzameld in dit proefschrift, toch als

zeer nuttig zullen ervaren. Ook jullie input had ik niet kunnen en willen missen. Dick en Elizabeth wil ik bedanken voor de samenwerking die heeft geresulteerd in de totstandkoming van hoofdstuk 5 en Steve voor de statistische adviezen bij hoofdstuk 3. In English what has been said in Dutch already: Elizabeth thank you for your co-operation resulting in chapter 5 and Steve without your help the statistics in chapter 3 would have taken me an awful lot longer to figure out. Mijn collega's bij "botgen" zou ik willen vergelijken met de altijd vrolijke en behulpzame Ewok's. Dus Leónie, Judith, Diana, Sangita, Mark, Joost, Mohamed, Salah en van langer geleden Wim, Sandor, Ton, Vered en Carlos BEDANKT. Dankzij jullie was de sfeer op de werkplek goed en dat maakte het erg plezierig om iedere dag weer te komen. Vergelijk het een beetje met het eindfeest op de bosmaan Endor. Gerrit, jij wordt Chewie, de Wookie, de oudste en sterkste van het stel. Ik heb zelden iemand ontmoet die groenere vingers heeft dan jij. Je hebt heel wat te stellen gehad met mijn soms wel erg krakkemikkige planten maar je hebt ze wel altijd goed verzorgd. Anita, ik kan helaas geen figuur voor jou bedenken, waarschijnlijk omdat ze in de films nauwelijks eten en drinken en daar dus niemand nodig hebben voor de vaat. Het maakt jou echter niet minder belangrijk want je neemt ons allemaal veel werk uit handen en aan leuke verhalen tijdens een rookpauze ook geen gebrek. Admistratieve hulp wordt geboden door Sei Taria, hetgeen in mijn geval om twee personen gaat: Aafke en Corrie E. Soazig Luke n'avait pas un "apprentice" alors pas un role dans le Film Star Wars pour toi mais un grand merci pour l'aide en "mapping" *grs*.
Last but not least alle vrienden en familie met speciale aandacht voor de stamgasten, Wilfried en Heleen die ik de rol van de Gungans toedeel, een vrolijk volkje waar je een hele hoop lol mee kunt beleven. En Karul, in dit geval mag jij dan de rol van JarJar op je nemen. Uitzondering wil ik ook nog maken voor Han Solo, Luke's trouwste vriend, Ivo bedankt. Juud, je mag kiezen, Eirtaé of Rabé, eigenlijk dienaressen van Queen Amidala maar je hebt mij vaak geholpen, al was het maar door voor de zoveelste keer te koken omdat ik weer alleen oog had voor mijn proefschrift. Verder denk ik dat we de beste living together apart relatie hebben die een mens kan hebben, waarvoor veel dank. Nelis, kapitein Panaka, hoofd veiligheidsdienst omdat je van boven waakt over mij. Flore, jij bent Luke's zus, princess Leia, je weet wel die met die rare knotjes. Een vriend heeft ze niet, hoewel Han Solo zeker een oogje op haar heeft. In mijn Star Wars epos kan dat niet want Joost en Ivo kunnen niet dezelfde rol vervullen. Geen Luke zonder zijn ouders, mama, jij de rol van Queen Amidala en papa, jij die van Anakin Skywalker. Ik ben zeer dankbaar voor alle steun en liefde die ik van jullie allemaal heb mogen ontvangen, of zoals ze in de film zeggen: may the Force be with you…….