An analysis of seed longevity in Arabidopsis using modifiers of seed maturation mutants

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Zusammenfassung

Samen gewährleisten das Überleben von Arten und damit den Erhalt von genetischen Ressourcen. Die Persistenz von Samen ist eine komplexe quantitative Eigenschaft, deren Ausprägung von Umweltbedingungen während der Bildung, Ernte und Lagerung der Samen abhängt. Zusätzlich wird die Samenpersistenz von der Samenstruktur, Makromolekülen und chemischen Verbindungen beeinflusst, die als Schutz des Embryos fungieren.

Die Identifizierung von Einflussfaktoren, die zu einer Verbesserung oder Reduktion von Persistenz bei Samen führen, ist dabei für die genetische Analyse notwendig. Wildtyp Samen der Modellpflanze *Arabidopsis thaliana* sind mehrere Jahre lebensfähig, weshalb die Analyse von Samenpersistenz einen zeitaufwändigen Prozess darstellt. Um dieses Problem zu umgehen, wurden die Samenentwicklungsmutanten *abi3-5* und *lec1-3* verwendet, in denen ein beschleunigter Alterungsprozess stattfindet. Diese Mutanten bieten damit einen sensibilisierten genetischen Hintergrund, vor welchem der Einfluss von Samenpersistenz regulierenden Genen beschleunigt evaluiert werden kann.

Die natürliche Variation von Samenpersistenz in Arabidopsis wurde genutzt, indem *abi3-5* und *lec1-3* mit verschiedenen Ökotypen gekreuzt und Nachkommen selektiert wurden, die eine verbesserte Persistenz aufwiesen. Drei Modifikator Linien mit dem stärksten Effekt wurden selektiert. Eine der Linien wies eine Introgression des Ökotyps Seis am Schlern in *abi3-5* auf, zwei Linien eine Introgression von Shahdara in *lec1*-Hintergrund. Diese Linien wurden mit *abi3-5* und *lec1-3* rückgekreuzt, um den Einfluss des jeweiligen Ökotypen-Genoms zu reduzieren und die Modifikatoren zu kartieren. Das Profil des Proteoms der Modifikator-Linien wurde in Zusammenhang mit Samenpersistenz untersucht. Dabei konnte gezeigt werden, dass die Modifikator-Linien von Shahdara, unabhängig von LEC1, die Expression von Samenspeicherproteinen aktivieren.

Zusätzlich wurden vier *abi3-5* Supressionsmutanten untersucht, die aus einem Mutagenisierungsdurchmusterung hervorgingen. In diesen Linen war eine Korrelation von oxidativen Schäden und Samenpersistenz nachweisbar. Die stärkste Supressormutante (*suppressor of abi-5*, *sua*) invertierte alle *abi3-5* Phänotypen. *SUA* wurde

mittels Fein-Kartierung und Karten-gestützter Klonierung identifiziert und kodiert ein RNA-bindendes Protein. Der Suppressionseffekt von *sua* trat allerdings nur spezifisch in Kombination mit dem *abi3-5*-Allel auf und nicht mit anderen *abi3* Allelen. Mittels immunologischer Analyse wurde gezeigt, dass *abi3-5*-Samen eine verkürzte Form des abi3-Proteins enthielten, wohingegen Samen der Doppelmutante *sua-1 abi3-5* ABI3-Protein in minimal reduzierter Größe zur Volllänge aufwiesen. Die Analyse der Transkripte zeigte, dass die *sua* Mutation das Spleißen eines kryptischen Introns in *ABI3* verursacht, wodurch eine Spleißvariante akkumuliert, die zu einer Reparatur der *abi3-5* Mutation und zur Bildung einer verkürzten, aber funktionellen ABI3-Isoform führt. Das *SUA*-Gen ist nicht direkt in der Samenpersistenz involviert, sondern spielt eine Rolle in den Prozessen des mRNA-Metabolismus.

Abstract

Seeds ensure the survival of most land plant species and the conservation of their unique genetic resources. Seed longevity is a quantitative trait that depends on environmental conditions during formation, harvest and storage of seeds and on structures, macromolecules and chemical compounds that protect the embryo. Seed longevity is consequently a complex genetic trait to dissect. Its study requires the identification of factors that result in an improvement or in a reduction of seed longevity. Wild-type seeds of the model plant Arabidopsis remain viable for several years, which makes the study of longevity a time consuming process. An approach to overcome this problem makes use of the seed developmental mutants *abi3-5* and *lec1-3*, that cause rapid seed deterioration. These mutants provide a sensitized genetic background in which the effects of genes influencing longevity can be faster evaluated.

The Arabidopsis natural variation for longevity was exploited by crossing several Arabidopsis accessions with *abi3-5* and *lec1-3* mutants and subsequent selection of lines with improved longevity in the progeny. As a result, various introgression lines carrying natural modifiers alleles were identified. The three natural modifier lines with the strongest effects were selected. One had an introgression of the Seis am Schlern accession in *abi3-5* background and two had different introgressions of the Shahdara accession in *lec1-3* background. These lines were backcrossed with *abi3-5* or *lec1-3* to reduce the contribution of wild-type accession's genome and to map the modifiers. The seed proteome profiles of modifier and mutant lines were studied in relation to longevity. This analysis revealed that the two modifiers from Shahdara could activate the expression of most seed storage proteins in a LEC1-independent way.

In addition, four *abi3-5* suppressor mutants derived from a mutagenesis screen were studied. In these lines the level of oxidative damage was correlated with seed longevity. The strongest suppressor, *suppressor of abi3-5* (*sua*), reverted all of the *abi3-5* mutant phenotypes. Fine mapping and map based cloning revealed that *SUA* encodes an RNA binding protein. Interestingly, *sua* only suppressed the *abi3-5* allele but did not affect other *abi3* alleles. Immunological analysis revealed that *abi3-5* seeds contain a truncated abi3

protein which is restored to nearly full length ABI3 protein in the *sua abi3-5* double mutant. Analysis of transcripts revealed that the *sua* mutation causes the splicing of a cryptic intron in *ABI3* and the accumulation of a splice variant that repairs the *abi3-5* mutation and results in a shorter but functional version of the ABI3 protein. The *SUA* gene is not directly implicated in seed longevity, but participates in mRNA metabolism processes.

Index

1 Introduction

1.1 Seed longevity

Seeds are reproductive structures that ensure the survival of most land plant species. Seeds of angiosperms and gymnosperms originate from ripened ovules which in most cases, following fertilization, develop into the embryo and the surrounding protective tissues. Key functions of seeds are the nourishment and protection of the embryo and the dispersal to new locations. In the majority of plant species a programmed desiccation phase occurs as the final stage of seed development. Seeds that dry at maturity and desiccate to less than 5% moisture content on fresh weight basis are termed orthodox. The mature dry seeds of many plant species are characterized by dormancy, which is the capacity to remain quiescent when the conditions are adequate for germination but not for seedling establishment (Baskin and Baskin, 1998). This is a strategic choice for reproductive success because it prevents germination at the wrong season, for instance during a few humid days in the middle of a dry summer. In addition, dormancy causes the staggering of germination of seeds from the same generation, thus limiting the competition between sibling plants (Nilsson et al., 1994). After dispersal in natural environments, but also during storage in controlled environments, orthodox seeds must maintain sufficient living tissue in order to be capable of germination when dormancy is lost and the environmental conditions concerning water availability, temperature and light are suitable.

Evolution endowed orthodox seeds with structures, enzymes and molecules that protect the embryo from the deteriorative effects of aging and slow-down the inexorable and irreversible processes that eventually result in the death of quiescent seeds. The speed with which viability declines varies among seed populations and certain genotypes deteriorate slower than others. Even within a genotype the storage potential of individual seed lots varies, and even within a seed lot individual seeds differ in their storage potential (Delouche, 1973; Walters et al., 2005).

Seed deterioration is detrimental to agricultural production, as it can reduce crop performance. Furthermore it undermines the conservation of the germplasm of

endangered and cultivated species, with little or no use in nowadays agriculture, but holding unique and irreproducible genetic resources.

Seed longevity refers to the life span of seeds and is determined by genetic factors (Miura et al., 2002; Clerkx et al., 2004a, b), by the interaction with the environment during plant growth and by deteriorative events that occur prior to or during storage (Bewley and Black, 1994). The model plant Arabidopsis can be considered as a reference species for the genetic and molecular dissection of this trait because it yields orthodox seeds with features common to many other plant species. In addition, its genome sequence is fully available and there is ever increasing information accessible about its physiology. Furthermore, Arabidopsis mutants with seed longevity phenotypes are available (Debeaujon et al., 2000; Clerkx et al., 2004a) and a collection of Arabidopsis accessions from many parts of the globe provides an additional large source of genetic variation that can be exploited for genetic studies.

1.2 Factors affecting seed longevity

Reduction of longevity is caused by the combined effects of lesions to macromolecules that accumulate during storage and the progressive inability to repair cellular damage (McDonald, 1999). Although deterioration reactions occur in the dormant seed, the effects are most often detected when conditions for germination are provided. Upon imbibition, damaged seeds can germinate slowly and with reduced vigour, exhibit abnormalities or, in the most severe cases, fail to germinate due to insufficient living tissue to carry out the processes leading to the seedling establishment. Loss of viability is generally caused by aging, and proceeds faster at high relative humidity and temperature, but it can also be an effect of the damage sustained during drying. The viability of a seed population is usually expressed as the percentage of individuals that can germinate, assuming that they are non-dormant.

One of the main causes for seed deterioration is the structural modification of macromolecules or the alteration of their distribution within cells (Black et al., 2006). Phase change of the polar membrane lipids, protein denaturation and the formation of sucrose crystals can damage the embryo cells. These processes depend on the storage conditions, particularly temperature and moisture availability. These two physical factors are directly

connected with seed deterioration during controlled storage and can be implemented in models for predicting the longevity of a seed population (Ellis and Roberts, 1980).

Spontaneous chemical reactions can also participate to the deterioration of stored seeds. Hydrolytic reactions occurring at high humidity storage can result in the scission of lipids and starches in free fatty acids and sugars, which in turn induce Maillard reactions and a cascade of oxidizing reactions (Murthy and Sun, 2000). Elevated moisture content can also trigger enzymatic reactions directed to the catalysis of storage reserves and imbalanced respiratory metabolism, resulting in the formation of partially reduced oxygen molecules, reactive oxygen species (ROS) and free radicals. Spontaneous oxidative reactions can also occur during dry storage in the presence of oxygen, which is able to penetrate seed tissues and acts to the detriment of unsaturated substrates such as the double bonds of fatty acids. These reactions are catalyzed by metals, heat and light and lead to the production of peroxides, extremely reactive molecules that are easily degraded to form the hydroxyl radical, which can set off an auto-oxidative cascade. Particularly sensitive targets of such damage are the nucleic acids (Britt, 1996). Seed deterioration damage can also occur at very low moisture content. During seed desiccation the removal of water causes mechanical strain and removal of the protective hydration shell of molecules.

1.3 Protection of the seed against deterioration

Orthodox seeds have evolved strategies to resist biotic and abiotic factors causing deterioration. Protective structures around the embryo and storage tissues are formed upon fertilization of angiosperms. The integuments of the ovule develop into the seed coat, a maternal tissue also called testa, which provides protection to mechanical damage and restricts water and oxygen uptake. Apart from this physical barrier the embryo can rely on a wide range of chemical compounds that establish desiccation tolerance: the mechanism enabling organisms to survive water loss. Molecules that prevent desiccation damage are accumulated during seed formation, and include non reducing sugars, oligosaccharides, stress proteins such as late embryogenesis abundant proteins (LEAs) and heat shock proteins (HSPs). These molecules protect the phospholipids bilayer of membranes, proteins and nucleic acids from the deleterious removal of water.

Non-reducing sugars and oligosaccharides accumulate at high concentration in the cytoplasm of embryo cells, where they induce the formation of a glass when the moisture content decreases to about 5% (Black and Pritchard, 2002). The extreme viscosity in the seed cellular compartments limits the mobility of molecules and consequently reduces damage (Buitink et al., 2000). The formation of such a glassy state leads to metabolic quiescence and at the same time preserves the functionality of macromolecular structures. Low temperatures and moisture content promote the glassy state, enhancing the longevity of seeds during storage.

The LEA proteins are highly hydrophilic proteins that accumulate in all cell compartments, particularly in the cytoplasm and the nucleus. They provide an interface between membranes and cytoplasm and stabilize macromolecules during drying (Delseny et al., 2001). The HSPs are ubiquitous but distinctively regulated by developmental cues and stress. They are abundant in desiccated seeds and decrease rapidly shortly after germination, just like the LEAs. They function as molecular chaperones (Wehmeyer and Vierling, 2000) and interact with target proteins to minimize the chance that hydrophobic regions illegitimately interact within the protein or with neighboring proteins, thus preventing protein denaturation and aggregation.

To cope with free radicals and ROS the efficiency of antioxidant defenses increases in the maturing orthodox seeds and energy consuming and producing processes are tightly controlled. Enzymes involved in the regeneration of molecular antioxidants, like superoxide dismutase, catalase and peroxidase are highly expressed. Furthermore, water soluble (glutathione, ascorbate) and lipid soluble (tocopherols, quinone, phenols) molecules that can scavenge ROS and the propagation of free radicals induced damage are accumulated.

1.4 Seed maturation and desiccation tolerance in Arabidopsis

In Arabidopsis a seed takes on average 16 to 22 days to fully develop. In the first seven to ten days after double fertilization the diploid embryo forms and acquires the basic architecture of the plant, after which it begins to expand as storage compounds are accumulated. In this phase, the forming Arabidopsis seeds start to exhibit the features that will lead to desiccation tolerance. During this process, which is called maturation, cells

initially expand and accumulate proteins, oils and sugars, subsequently water is progressively lost, the metabolism slows down and the embryo prepares for germination but remains dormant. Lipids in the form of triacylglycerols are synthesized and stocked in oil bodies in the cotyledons. Storage proteins, particularly 12S Globulin and 2S Albumin, but also LEAs and HSPs, are produced in large amounts. During the last days of maturation sucrose and polysaccharides (like stachyose and raffinose) are also accumulated and are supposed to contribute to the establishment of desiccation tolerance (Figure 1.1; Baud et al., 2002). At the end of the seed filling period, orthodox seeds acquire the ability to withstand severe stresses that would be lethal during the vegetative plant life cycle.

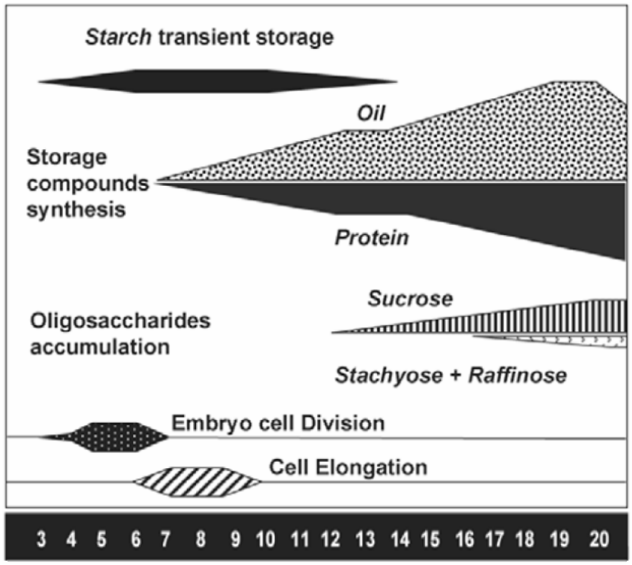


Figure 1.1: Representation of the major physiological changes in the Arabidopsis seed composition during development. The black bar at the bottom is the time scale in days after fertilization (DAF). After ten DAF the embryo morphogenesis is completed and the maturation phase takes place. The seed gradually desiccates and, at approximately 20 DAF becomes dry and ripe. (Figure adapted from Baud et al., 2002)

1.5 Control of seed development in Arabidopsis

Seed development and maturation are regulated by the concerted action of several signaling pathways, integrating information from genetic programs and from both hormonal and metabolic pathways (Wobus and Weber, 1999). Abscisic acid (ABA) plays a central role during seed maturation by preventing precocious germination and reserve mobilization. It is involved in the metabolism of reserve deposition, acquisition of desiccation tolerance and dormancy (Finkelstein et al., 2002). ABA is first produced in maternal tissues (Frey et al., 2004) and subsequently in the embryo. ABA accumulation peaks during maturation of Arabidopsis seeds and ABA signaling is particularly connected with the expression of four master regulator genes with partially redundant

functions: *LEAFY COTYLEDON1* (*LEC1*), *LEC2*, *FUSCA3* (*FUS3*) and *ABSCISIC ACID INSENSITIVE3* (*ABI3*). *LEC1* encodes a protein with sequence similarity to the HAP3 subunit of CCAAT binding factor homologues in other organisms and is part of an oligomeric transcriptional activator (Lotan et al., 1998). *LEC1*, together with *LEC2* and *FUS3*, is required for desiccation tolerance, synthesis and accumulation of storage reserves and inhibition of germination. Furthermore it coordinates the embryo morphogenesis and maturation and confers embryogenic competence to cells (Harada, 2001; Gaj et al., 2005). *LEC2*, *FUS3* and *ABI3* encode for plant specific transcription factors containing the conserved B3 DNA binding domain (Giraudat et al., 1992; Luerssen et al., 1998; Stone et al., 2001) that physically binds to the Sph/RY cis-element in the promoter region of seed specific genes (Ezcurra et al., 1999; Reidt et al., 2000). ABI3 is a main transducer of the ABA hormone signal, necessary for the expression of maturation genes, storage protein genes (Parcy et al., 1997; Kroj et al., 2003), *LEA* genes (Giraudat et al. 1992), genes with antioxidant functions (Haslekås et al., 2003) and *HSP* genes (Kotak et al., 2007). ABI3 shares highly conserved protein domains with all the homologues cloned so far from other plant species, designated A1, B1, B2, and B3, starting from the N-terminal (Giraudat et al. 1992; Suzuki et al. 1997; Figure 1.2).

Figure 1.2: Schematic representation of the ABI3 transcription factor (720 aa). A1 represents the acidic activation domain, while B1, B2 and B3 are basic domains necessary for the regulation specificity of seed developmental genes.

The A1 domain is a functional acidic transcriptional activation domain (McCarty et al., 1991). The B1 domain is involved in the physical interaction with the seed specific transcription factor ABSCISIC ACID INSENSITIVE5 (ABI5) (Nakamura et al., 2001). The B2 domain is responsible for the ABA dependent activation of ABA regulated genes through the ABA-response *cis*-acting element (ABRE) (Hill et al. 1996; Bies-Etheve et al., 1999; Ezcurra et al., 2000), and facilitates the interaction with bZIP transcription factors (Hill et al., 1996) such as ABI5. Finally, the B3 domain has been shown to bind DNA in vitro (Suzuki et al., 1997; Nag et al., 2005) and is also found in other plant specific transcription factors like LEC2 and FUS3 (Riechmann et al., 2000). Mutations in any of the

LEC1, *LEC2*, *FUS3* or *ABI3* genes affect multiple processes including the accumulation of storage proteins and the acquisition of desiccation tolerance (Koornneef et al., 1984; Nambara et al., 1995; Meinke, 1992; Parcy et al., 1997; Vicient et al., 2000; Stone et al., 2003; Kroj et al., 2003; West et al., 1994). Recent studies combining *lec1*, *lec2*, *fus3* and *abi3* Arabidopsis mutants with transgenic reporter gene lines and overexpression lines of the same transcription factors unraveled the interactions within this main genetic network regulating seed maturation (To et al., 2006; Figure 1.3)

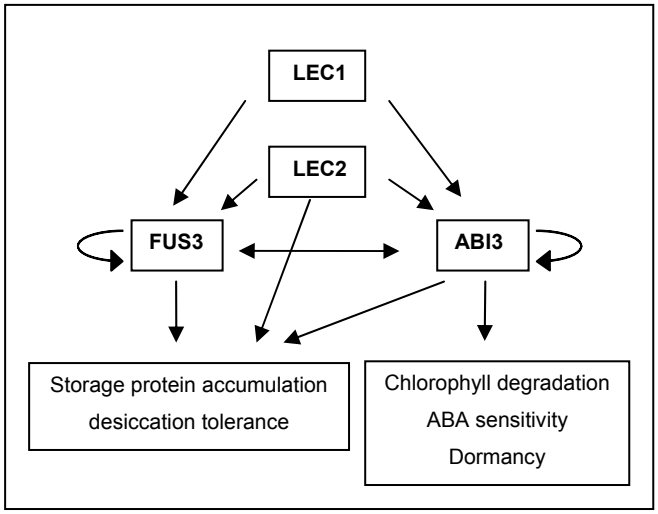


Figure 1.3: schematic representation of the relationship between key members of the genetic network regulating seed maturation. *LEC1* is expressed during embryo morphogenesis, while *LEC2*, *FUS3* and *ABI3* are expressed during maturation. The arrows indicate direct control on gene expression or on developmental processes. This figure is adapted from To et al. (2006).

In addition to these four central transcription factors and their direct targets, many more genes exist that exert at least a marginal role in maturation and desiccation tolerance. Each of them can contribute to seed longevity, but due to redundancy and the major influence of the environment during and after seed formation the effects of single mutants can be very small and scarcely detectable. Despite of this, in the past years several genes that can affect seed longevity have been identified including bZIP transcription factors transducing the ABA signal during seed formation (Jakoby et al., 2002; Bensmihen et al., 2005). Furthermore, genes implicated in the formation of the testa (Debeaujon et al., 2000), in the resistance to desiccation (Wehmeyer and Vierling, 2000; Hoekstra et al., 2001), in the protection against oxidative damage (Sattler et al., 2004) and in the regulation of non reducing sugars metabolism (Gomez et al., 2005, Ramon et al., 2007) have also been correlated with seed longevity.

RNA metabolism plays a crucial role in the regulation of gene expression during all stages of plant growth, including seed development. Post-transcriptional gene regulation

includes pre mRNA splicing, capping, polyadenylation, mRNA transport, stability and translation (Simpson and Filipowicz, 1996). In these processes, regulation is either achieved directly by RNA-binding proteins (RBPs) or indirectly, whereby RBPs modulate the function of other regulatory factors. There are several indications that the ABA signal transduction pathway is strongly influenced by RNA metabolism. The flowering time controller and ABA receptor gene *FCA* is subject to alternative splicing (Quesada et al., 2005; Razem et al., 2006). In addition the ABA hormone itself has been shown to regulate the abundance of spliceosomal genes (Hoth et al., 2002; Raab and Hoth, 2007), and the induction of a micro RNA that in turn modulate the expression of MYB transcription factors (Lu and Fedoroff, 2000; Reyes and Chua, 2007). Alternative splicing of *ABI3* homologues in wheat and other cereal species determines the grain quality and controls pre-harvest sprouting (McKibbin et al., 2002). RNA metabolism can therefore influence the seed storage compounds abundance and composition.

1.6 Techniques for seed longevity assays

As mentioned in the former paragraphs, seed longevity depends on environmental conditions during seed formation, harvest and storage and is controlled by probably many genes. Consequently it is a complex genetic trait that is difficult to analyze genetically. The variability that characterizes seed longevity can hamper a clear and efficient evaluation of phenotypes from alleles with weaker effect. Orthodox seeds like those of Arabidopsis generally survive storage for more than five years, and small but significant differences in the longevity of two genetically different seed batches can take several years before clearly emerging. To cope with this problem, strategies for the artificial acceleration of seed aging have been developed (Tesnier et al., 2002) and are currently employed as a method to evaluate seed quality. The Controlled Deterioration Treatment (CDT) consists in storing the seeds in controlled environmental conditions at high relative humidity and temperature. Such treatment causes a quick burst of oxidative damage that mimics natural deterioration processes which take place during long periods of storage (Khan et al., 1996). At regular time intervals the survival percentage of the seeds is estimated with germination tests.

1.7 Research approach

Studying the molecular and genetic control of seed longevity requires the identification of novel alleles that either result in improved or reduced seed quality. The approach followed here makes use of mutants that deteriorate very quickly after harvest, and therefore provide a sensitized genetic background where the effects of alleles influencing longevity can be faster evaluated (Clerkx, 2004c). In Arabidopsis, loss of function mutants of the key regulators of seed development *LEC1* and *ABI3* produce seeds that lose their viability during the first few weeks after harvest, whereas the wild-type seeds can be stored for several years (Meinke, 1992; Nambara et al., 1995). The *lec1-3* allele is a homeotic mutation that causes abnormal vascular tissues in the embryo, the formation of trichomes on cotyledons and the skipping of the maturation programs, which leads to severe intolerance to desiccation of mature seeds. Plants affected by this mutation can grow normally but yield seeds that are shriveled and flattened due to a lack of protein and oil reserves, tend to be viviparous and accumulate anthocyanins in the embryos (Figure 1.4). The *abi3-5* mutation specifically prevents proper seed maturation including the acquisition of desiccation tolerance. This allele was originally isolated in a mutagenesis screen for ABA insensitivity at germination. *abi3-5* seeds are non-dormant and can germinate in the presence of high concentrations of the hormone, furthermore they retain chlorophyll after senescence and remain green during the first months of storage (Figure 1.4).

Figure 1.4: The seed phenotype of the developmental mutants *lec1-3* (left) and *abi3-5* (right), compared to Landsberg *erecta* (middle). Some *lec1-3* seeds display premature radicle protrusion, a characteristic of vivipary.

1.7.1 Natural modifiers of *lec1-3* and *abi3-5*

The plant species Arabidopsis, which grows over a wide geographic range and in different habitats, has evolved various strategies for ecological adaptation and therefore shows natural variation for many traits (Shindo et al., 2007). Quantitative trait loci (QTL) mapping between different parental accessions has been of great importance in understanding complex traits and explaining differences in gene function and genetic pathway structures (Koornneef et al. 2004). Recombinant inbred lines (RILs) and introgression lines, also called near isogenic lines (NILs) are an important resource to identify genes that underlay natural variation for different traits. These lines can serve as starting material for fine-mapping and ultimately cloning the genes underlying natural variation. QTLs are likely to play a role in determining differences in longevity between seeds from accessions occupying specific environmental niches. Previous studies revealed genetic variation for Arabidopsis seed traits such as dormancy and longevity (Alonso-Blanco et al., 2003; Bentsink et al., 2000; Clerkx et al., 2004b).

The analysis of recombinant lines, derived from crosses between different Arabidopsis accessions and the *abi3-5* or *lec1-3* mutants in L*er* background, was used to identify alleles that enhance longevity in the mutant genetic background. Such alleles, so called natural modifiers, have been detected in introgression lines with chromosomal segments from exotic accessions in homozygous *abi3-5* or *lec1-3* mutants (Clerkx 2004c). The increased seed longevity of these lines can be due to specific interaction between modifiers and the mutant background alleles, but can also be due to the modification of other pathways of seed development. In the latter case the effects of the natural modifiers would be independent from the mutants and would also be detectable in NILs with wild-type backgrounds.

1.7.2 Suppressor modifiers of *abi3-5*

Another approach for the identification of genes influencing seed longevity is mutagenesis. As mentioned above mutants would be more easily identified in a genetic background characterized by reduced seed viability. A mutagenesis screen in such a genotype would allow the identification of enhancer or suppressor mutations (Page and Grossniklaus, 2002). This approach was used to identify an enhancer mutant of the weak

abi3-1 allele, *green seed* (*grs*), which affects the pathway of chlorophyll degradation in maturing seeds (Clerkx et al., 2003). The *grs abi3-1* double mutant displays an enhancement of some but not all the phenotypes of *abi3-1*. The many pleiotropic aspects of the *abi3-5* mutant (paragraph 1.7) suggest that it could also be used in a suppressor mutagenesis screens to dissect the ABI3-dependent genetic pathway of seed maturation. This approach was followed and led to the identification of double mutant lines displaying a partial reversion of the poor seed longevity of *abi3-5* (Clerkx, 2004c). The novel mutant alleles acted as suppressors of *abi3-5*, which might affect a subset of downstream targets of ABI3, or be part of overlapping genetic networks that govern seed maturation.

1.8 Scope of this thesis

The aim of this thesis was to better understand seed longevity and the processes that reduce it. The effects of the environment on the seeds during storage, and the role of specific genes active during seed maturation were the topics of interest.

The *lec1-3* and *abi3-5* mutants were chosen as a tool for studying longevity because of their effects on seed development. The modifier alleles of these two mutants, characterized by slower seed deterioration, offered the possibility of studying specific aspects of the trait.

One objective was to confirm the phenotypes of the modifier lines by evaluating their seed longevity in comparison to the *lec1-3* and *abi3-5* mutants in L*er* background. An analysis of the seed physiology of the modifiers, including proteome profiling, was required to explain the increased longevity. Mapping experiments were also necessary in order to assess the genetic determinism of the modifiers.

For the strongest suppressor of *abi3-5* the focus of research was to understand how the suppression phenotypes were achieved at the molecular level. The functional characterization and the cloning of this modifier were necessary to unravel its relationship with *ABI3* and with seed development in general.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

The chemicals used in this work were purchased from the following companies: Biorad (Hercules, USA), Carlo Erba (Milan, Italy), Difco Laboratories (Detroit, USA), Invitrogen (Karlsruhe, Germany), MBI Fermentas (St. Leon-Roth, Germany), Merck (Darmstadt, Germany), Promega (Mannheim, Germany), Roche (Mannheim, Germany), Sigma (Deisenhofen, Germany) and Carl Roth (Karlsruhe, Germany).

2.1.2 Antibiotics

All antibiotics were purchased from Duchefa (Haarlem, Netherlands). The stock solutions were frozen at – 20°C in small aliquots

Table 2.1.1: Antibiotics and working concentrations

Name (Abbreviation)	Stock Solvent	[mg/l] in Selection Medium
Ampicillin (Amp)	H_2O	100
Carbenicillin (Carb)	Ethanol	50
Chloramphenicol (Cm)	Ethanol	25
Gentamycin (Gent)	H_2O	10
Hygromycin (Hyg)	H_2O	50
Kanamycin (Kan)	H_2O	50
Rifampicin (Rif)	Methanol	50
Spectinomycin (Spec)	H_2O	100
Tetracyclin (Tet)	H_2O	10

2.1.3 Buffers and Culture Media

In general, buffers and media for laboratory work were prepared according to Sambrook and Russel (2001). For some applications special solutions were prepared as described below:

High salt solution for RNA precipitation:	
Sodium Citrate	1.2 M
NaCl	0.8 M
FOX reagent:	
Ammonium ferrous sulfate	250 μM
Xilenol Orange	100 μM
H_2SO_4	25 mM
Methanol	90% (v/v)
GUS staining buffer:	
Triton X-100	0.2% (v/v)
$NaPO_4$	50 mM, pH 7.2
$K_4Fe(CN)_6$* H_2O	2 mM
$K_3Fe(CN)_6$	2 mM
X-Gluc	2 mM
Induction Medium:	
$MgCl_2$	10 mM
MES	10 mM, pH 5.6
Acetosyringon	150 μM
Seed Protein Extraction Buffer (A):	
Urea	8 M
Triton X-100	0.2% (v/v)
Sarkosyl	0.2% (w/v)
Tris-Cl	100 mM, pH 7.5
Seed Protein Extraction Buffer (B):	
Urea	7 M
Thiourea	2 M
Triton X-100	0.2% (v/v)
CHAPS	58 mM
Tris base	14 mM
Tris-Cl	18 mM
Ampholytes	1% w/v, pH 3-10
Protein Precipitation Solution:	
Acetone	80% (v/v)
Trichloroacetic acid	20% (v/v)
DTT	1 mM

Lysis/Binding solution:	
Na_2HPO_4	1 mM
NaH_2PO_4	1 mM
NaCl	0.5 mM
Imidazole	20 mM
Blocking Solution:	
Tris-Cl	50 mM
NaCl	150 mM
Tween 20	0.25% (v/v)
Skim Milk	5% (w/v)
Germination Medium:	
Daishin agar	0.6% (w/v)
Murashige and Skoog Salts	0.1% (w/v)
HCl	to pH 5.7
Rescue Medium:	
Daishin agar	0.6 % (w/v)
Glucose	0.6% (w/v)
Murashige and Skoog Salts	0.1% (w/v)
HCl	to pH 5.7

2.1.4 Enzymes

All restriction enzymes were ordered from New England Biolabs® (Schwalbach/Taunus, Germany) or Roche (Mannheim, Germany)

Klenow fragment exo (MBI fermentas, Germany)

Lysozyme (Roche, Mannheim, Germany)

Platinum® *Pfx* DNA-Polymerase (Invitrogen, Karlsruhe, Germany)

Ribonuclease Inhibitor (Roche, Mannheim, Germany)

RNase H (Promega, Mannheim, Germany)

Superscript™ II reverse transcriptase (Invitrogen, Karlsruhe, Germany)

Taq DNA Polymerase (Invitrogen, Qiagen or Roche)

T4 DNA Ligase (Invitrogen, Karlsruhe, Germany)

2.1.5 Commercial Kits

Bio-Safe™ Comassie G-250 stain (Biorad, Hercules, USA)

BP-Clonase and LR-Clonase (Invitrogen, Karlsruhe, Germany)

First Strand cDNA Synthesis Kit (Invitrogen, Karlsruhe, Germany)

High Pure PCR Product Purification Kit (Roche, Mannheim, Germany)

HisTrap FF crude Kit (GE Healthcare, Germany)

Megaprime™ DNA Labelling Systems (Amersham Biosciences, Little Chalfont Buckinghamshire, England)

Miniprep™ Kit (Qiagen, Hilden, Germany)

Protein assay (Biorad, Hercules, USA)

RNAqueous RNA isolation aid (Ambion, Austin, USA)

RNeasy Plant Mini® Kit (Qiagen, Hilden, Germany)

Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen, Karlsruhe, Germany)

BACMAX™ Kit (EPICENTRE®, Madison, USA)

2.1.6 Oligonucleotides and plasmids

All the primers used in this work are listed in table X (polymorphic markers), Z (molecular cloning) and Y (*sua-1* finemapping and sequencing, genotyping of mutants), they were ordered from Invitrogen or Operon. The plasmids used for creating new constructs were provided by colleagues at the Max Planck Institute für Züchtungforschung, except for the pCR®-BluntII-TOPO® that was ordered from Invitrogen (Karlsruhe, Germany).

Table 2.1.2: Vectors used in this work

Vector	Purpose	Provided by
pAM-PAT 35S GW GFP	overexpression of GFP labeled proteins in plants	Sandra Noir (MPIZ)
pBAT B	binary vector for plant transformation	Joachim Uhrig (MPIZ)
pGreen 0229	binary vector for plant transformation	Fabio Fornara (MPIZ)
pCR®-BluntII-TOPO®	Direct cloning of PCR products	Invitrogen, Karlsruhe, Germany
pDONR™ 207	donor vector for the cloning of PCR products with the Gateway® technology	Invitrogen, Karlsruhe, Germany
pGWB3 GUS	Study of promoter activity in Planta with GUS reporter	Kazumi Nakabayashi (MPIZ)
pLeela GW	overexpression of cDNA in Planta	Joachim Uhrig (MPIZ)
pET-32	overexpression of His tagged proteins in *E. coli*	Christina Philipp (MPIZ)

2.1.7 Bacterial strains

For cloning purposes chemical transformation competent or electro competent DH5α strains of *Escherichia coli* were used (Hanahan, 1983). For plant transformation the chemical transformation competent GV3101 strain of *Agrobacterium tumefaciens* was used. In transformation experiments with the binary vectors pGreen 0229, pLeela Gw and pAM-PAT 35S GW GFP, Agrobacteria strains carrying respectively psoup or pMP90RK helper vectors were used (Koncz and Schell, 1986; Koncz et al., 1990; Hellens and Mullineaux, 2000).

2.1.8 Seeds and genotypes

Arabidopsis accessions: Landsberg *erecta* (L*er*), Cape Verde Islands (Cvi), Columbia (Col), Seis am Schlern (Sei), Shahdara (Sha), Eilenburg (Eil), Warschau (Wa). **Near isogenic lines** (NILs) and **natural modifier introgression lines** are listed in Tables 2.1.3 and 2.1.4.

Mutants: *glabra (gl) gl1* (Oppenheimer et al., 1991), *abscisic acid insensitive (abi) abi3-5* (Ooms et al., 1993; Bies-Etheve et al., 1999), *abi3-6* (Nambara et al., 1994), *abi3-4* (Giraudat et al., 1992), *leafy embryo cotyledons (lec) lec1-3* (Raz et al., 2001), *lec2-1*, *fusca (fus) fus3-1*(Holdsworth et al., 1999, Finkelstein et al., 2002), *transparent testa (tt) tt5* (Koornneef 1981, 1990). All these mutations are recessive morphological markers recognizable because they consistently alter the seed aspect, *tt5* has maternal effects. *abi3-1*, *abi3-7* (Ooms et al., 1993; Bies-Etheve et al., 1999), *sua-1* (identified in this work), *sua-2* (SALK T-DNA insertion line, Alonso et al., 2003), *sua-3* (GABI-KAT T-DNA insertion line, Rosso et al., 2003) could be tracked by specific PCR markers.

Table 2.1.3: List of near isogenic lines (NILs), all in L*er* background

Name	Introgression Position	Chromosome	Accession
LCN 3-12	between 8 and 23.5 Mb	3	Cvi
LCN 3-16	between 16 and 23.5 Mb	3	Cvi
LCN 5-8	between 8 and 20 Mb	5	Cvi
LCN 5-15	between 20.7 and 25 Mb	5	Cvi
LCN 5-19	between 24.7 and 26.8 Mb	5	Cvi
LShetb1	between 25.7 and 26.8 Mb	5	Sha
LShetb2	between 23.6 and 26.3 Mb	5	Sha
LShetb3	between 21 and 24.5 Mb	5	Sha
5b.17	between 21 and 26.8 Mb	5	Sha

Table 2.1.4: List of natural modifier introgression lines, all in L*er* background

Name	Introgression Position	Chromosome	Accession	Background Mutation
lec/Sha1	between 0 and 9 Mb	4	Sha	*lec1-3*
lec/Sha2	between 18 and 26.8 Mb	5	Sha	*lec1-3*
abi/Sei	between 0.6 and 10 Mb	5	Sei	*abi3-5*
abi/Wa	between 0 and 7 Mb	5	Wa	*abi3-5*
abi/Eil	Between 7.8 and 17.1 Mb	4	Eil	*abi3-5*

The NILs: LShetb1, LShetb2, LShetb3 and 5b.17 were provided by Bjorn Pieper (Max Planck Institut für Züchtungforschung). All the other genotypes except for *sua-2* and *sua-3* were obtained from the seed stocks in Wageningen University (The Netherlands) and provided by Maarten Koornneef.

2.1.9 Most useful software and websites

Analysis of sequencing results: DNASTAR, Version 7.0.0

Sequences analysis: GENE RUNNER, Version 3.05

In-silicon cloning: Clone Manager 7, Version 7.11

Services at the MPIZ: http://www.mpiz-koeln.mpg.de/

Universal web search: http://www.google.com

Arabidopsis information resource: http://www.arabidopsis.org/

Primer design: http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi

Bioinformatics tool: http://www.ncbi.nlm.nih.gov/

DNA sequences alignments: http://bioinfo.genopole-toulouse.prd.fr/multalin/

2.2 Methods

2.2.1 Condition of plant growth

All plants were grown on soil containing a mixture of substrate and vermiculite (3:1), in a greenhouse where the temperature was maintained close to 20°C. Sodium and mercury lamps integrated the natural photoperiod in order to provide long day conditions of 16 hours light throughout the year.

2.2.2 Crossings

The inflorescence of the pollen acceptor plant (mother plant) was cleared from open flowers, apical meristems and developing siliques, leaving just 2 to 3 immature and closed flower buds with immature anthers. With the help of a fine forceps and a loupe all the sepals, petals and stamens were removed, leaving the bare pistils. One or two anthers from recently opened mature flowers from the pollen donor (father plant) were picked with the clean forceps and rubbed on the prepared pistils of the mother plant, ensuring that enough pollen grains were stuck on the stigma. For the next 2 days the fertilized flowers were surrounded by cellophane in order to maintain high moisture during the fecundation.

2.2.3 Seed storage

Shortly after harvest seeds were sieved and stocked into small, non sealed plastic bags that were stored in the laboratory cupboards at room conditions. For long term storage the seed bags were put in a special room at 5°C and 30% relative humidity, or in the freezer at -80°C.

2.2.4 Selection of Modifier Lines

The selection of modifiers was performed as shown in figure 1, the selection procedure started in Wageningen University where Emile Clerkx and his colleagues crossed 20 Arabidopsis accessions, chosen for having a relatively short flowering time, with the

reduced seed longevity mutants *lec1-3* and *abi3-5*, in L*er* background (Clerkx 2004c). Five of the lines generated by the process were further analysed, namely: *abi*/Eil, *abi*/Sei, *abi*/Wa, *lec*/Sha1, *lec*/Sha2.

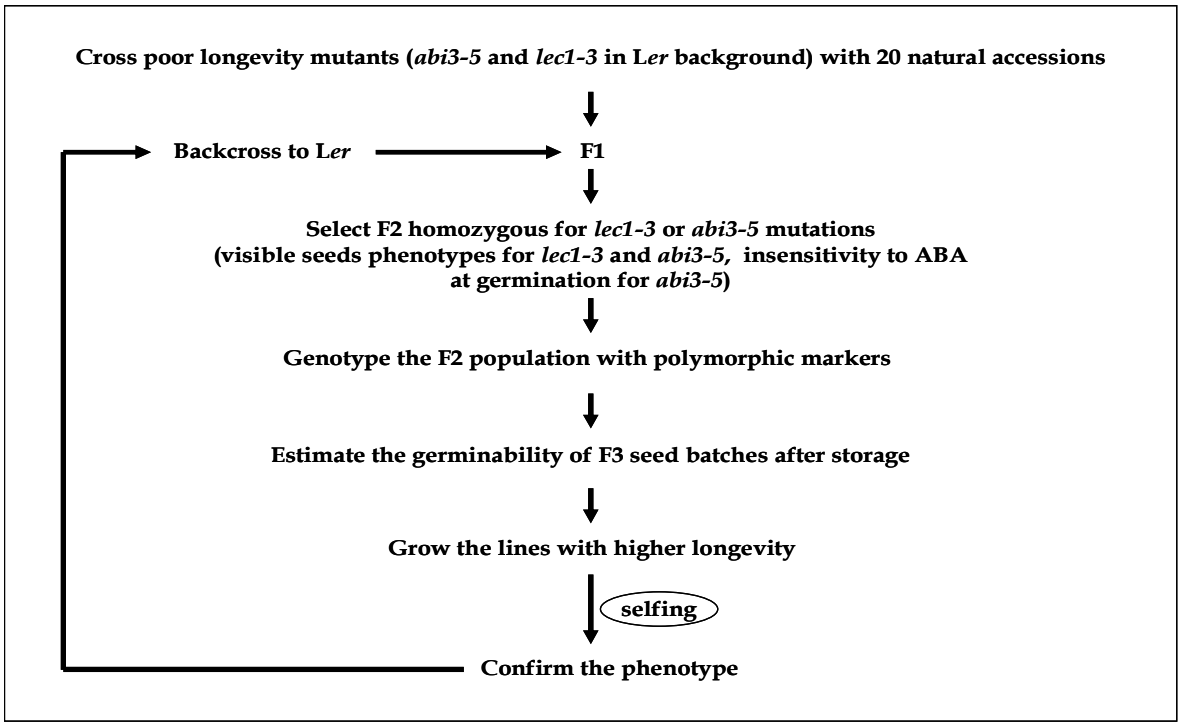


Figure 2.1: Selection strategy for the modifier lines

2.2.5 Controlled deterioration test

For accelerating the deterioration of the seeds and simulating the effects of aging, a controlled deterioration test was performed (Tesnier et al., 2002). Aliquots of approximately 300 seeds were put into open PCR tubes and incubated in sealed exicators at room temperature. In the bottom part of every exicator, below the tubes, different compositions of saturated salt solutions were placed, as described by Winston and Bates (1960). In this way different relative humidity conditions were created: 23% (Potassium Acetate) 43% (Potassium Carbonate), 69% (Potassium Iodide), 94% (Potassium Nitrate).

For the testing of wild-type and lines characterized by slowly deteriorating seeds, more drastic conditions were used. An exicator containing a saturated solution of Potassium Chloride, that generates an environment with 85% relative humidity, was placed into an incubator at 37°C. In these conditions a few days were sufficient to strongly reduce the viability of wild-type mature seeds.

2.2.6 Germination tests

For monitoring seed viability during storage we performed periodic assays on their ability to germinate. Approximately 100-150 seeds from each batch were sown into six cm wide plastic Petri dishes, on round filter papers (Macherey & Nagel, Düren, Germany) soaked with 580 µl demineralized water. Depending on the experiment a stratification treatment, consisting of 5 to 7 days incubation at 4°C in the dark, was provided. Then seeds were put into an incubator, in long day conditions (16 hours light at 25°C, followed by 8 hours darkness at 20°C). After one week of incubation the germinated seeds were counted with the help of a stereomicroscope (MZ6 from Leica, Germany). The fraction of seeds that germinated represented the viable seeds at the moment of sowing. In dormancy experiments, seed batches that were supposedly 100% viable were processed in the same way, but without providing any prior stratification treatments. In this case, after incubation, the non germinated seeds represented the fraction of dormant seeds. In the experiments for assessing the abscisic acid hormone (ABA) sensitivity, seeds were stratified and incubated as described above but on filter papers soaked with solutions of different ABA concentrations. With this test we could evaluate at which concentration germination was inhibited.

2.2.7 Longevity estimation

The germinability of the seeds was monitored periodically and plotted to a graph; the curves describing the loss of viability over time, and the steepness of their trend lines, gave an estimation of seed longevity.

2.2.8 Seed coat sterilization

Sterilizing seeds surface consisted of two washing steps: the first with 70% ethanol and the second with pure ethanol, afterwards the seeds/ethanol suspension was poured on filter paper and dried on the sterile bench. This procedure was applied to seeds to be sown on solid Germination Medium, completed with sugars for seedling rescue or with antibiotics for transgenic seedlings selection (see 2.2.23).

2.2.9 Rescue of mutant seedlings

For the propagation of lines and populations of plants characterized by poor seed viability, like those carrying severe mutations such as *lec1-3* or *abi3-5*, was allowed on solid Rescue Medium plated into Petri dishes. Once the seedlings were sufficiently established, generally 10 days after germination, they were transferred to soil. During the first days of greenhouse growth the seedlings were sheltered from direct light.

2.2.10 FOX method

To determine the degree of seed lipid oxidation 800 to 1000 seeds were counted for each line, and directly ground in a mortar with liquid nitrogen. As soon as a powder was obtained, 450 μL of extraction solvent mixture of isopropanol and chlorophorm 9:1 was added. Seed extract was then collected into an eppendorf tube and centrifuged at 14000 rpm for 5 minutes. 200 μL of the supernatant was mixed with 1800 μL of FOX reagent (see 2.1.3), prepared according to the method described by Nourooz-Zadeh et al. (1995). The reaction mix was incubated for 30 minutes at room temperature in the dark. After that the absorbance of each sample was monitored at 532 nm with an Uvicon 810 Spectrophotometer. For estimating the peroxides concentration in the samples a standard curve was created. Seven aliquots of 200 μL of extraction solvent containing different concentrations of hydrogen peroxide ranging from 0 to 60 μM were mixed with the FOX reagent and measured as described before.

2.2.11 Seed chlorophyll content

For determining the seed chlorophyll content of the lines carrying the *abi3-5* mutation, between 500 and 600 seeds from each sample were ground in a mortar with liquid nitrogen. The resulting powder was then resuspended in 1 ml of 80% Acetone. After 5 minutes of centrifugation at 14000 rpm the supernatant was used for measuring absorbance at 663.6 and 646.6 with an Uvicon 810 Spectrophotometer (Kontron Instruments, Zurich, Switzerland).

Total chlorophyll concentration was calculated as described in Marr et al., (1995), as the sum of Chlorophyll a and Chlorophyll b.

Chlorophyll a (mg/L) = 12.25 * $A_{663.6}$ – 2.55 * $A_{646.6}$

Chlorophyll b (mg/L) = 20.31 * $A_{646.6}$ – 4.91 * $A_{663.6}$

2.2.12 DNA extraction

The equipment and buffers used for plant DNA extraction were from Qiagen (Hilden, Germany). A few leaf pieces per plant were put into collection microtubes, together with tungsten-carbide beads, tubes were sealed with collection microtube caps and frozen in liquid nitrogen. The tissue was destroyed with a mixer mill (MM300, Qiagen, Hilden, Germany). After that, the protocol for purification of total DNA from plant tissue using BioSprint workstation was followed according to the manufacturer instructions.

Alternatively, for small scale plant DNA extraction a simple protocol was used as described in Dellaporta et al., 1983.

DNA from *E. coli* was extracted from cultures grown in 2 to 5 ml Luria Betani (LB) media overnight at 37°C. The cell paste was treated with the Miniprep™ Kit from Qiagen (Hilden, Germany) following the manufacturer's instructions. For Bacteria Artificial Chromosome (BAC) isolation, cells from a large volume of culture (500 ml) were collected and treated with the BACMAX™ kit from EPICENTRE® (Madison, USA).

2.2.13 DNA purification

PCR amplified DNA was purified using High Pure PCR Product Purification Kit from Roche (Mannheim, Germany) or PeqGOLD Cycle Pure Kit from PEQLAB Biotechnologie GMBH, Germany. DNA fragments from agarose gels were purified using the QIAquick Gel Extraction Kit from Qiagen (Hilden, Germany).

2.2.14 Nucleic Acids quantification

The nucleic acids (DNA and RNA) were always quantified by using the nanodrop ND-1000 Spectrophotometer from PEQLAB Biotechnologie GMBH, Germany.

2.2.15 Mapping

Genetic mapping of segregating populations was done by using a number of polymorphic PCR markers, mostly simple sequence length polymorphism (SSLP), or cleaved amplified polymorphic sequences (CAPS). Most of the primers were designed for flanking short polymorphic sequences referring to the Monsanto Lansberg-Columbia polymorphisms database (https://www.arabidopsis.org/cgi-bin/cereon/). The markers were tested on genomic DNA from the required accessions, as they could often identify multiallelic polymorphisms. After PCR reaction, in the case of CAPS markers, the amplified products were digested with the appropriate enzyme and, like in the case of SSLP, separated on 3% agarose gels to detect the polymorphisms.

To increase the density of the selected lec/Sha1, lec/Sha2 and abi/Sei Modifier's genetic maps, after the 6th round of backcrossing and selfing, DNA samples were sent to the company Illumina® (San Diego, USA) that provided several new data about single nucleotide polymorphisms (SNPs).

2.2.16 Sequencing

DNA sequences were amplified by PCR or in bacterial strains, purified and quantified as described above. Sequencing was performed by the Automatische DNA Isolierung und Sequenzierung (ADIS Unit) at the MPIZ. The output data was analyzed with the DNASTAR software.

2.2.17 RNA extraction

RNA extraction from leaves was done with the RNeasy® plant mini kit from Qiagen (Hilden, Germany), following the manufacturer instructions.

For extracting RNA from siliques a more complex protocol was needed, since polysaccharides, oils and storage proteins present in this tissue affect the RNA purity.

About 20 mg of siliques was ground into fine powder in a mortar with liquid nitrogen. This material was then treated with the RNAqueous™ total RNA isolation kit from Ambion (Austin, USA), following the related instructions. In this way 100 µl of non pure

total RNA was obtained, the concentration was measured. The RNA solution was diluted with RNase free water up to 1 ml and precipitated by adding 250 µl of isopropanol and 250 µl of High Salt Precipitation Solution. After mixing and keeping the tubes on ice for 2 hours the RNA was recovered by centrifugation at 14000 rpm for 15 min at 4°C. The pellet was rinsed with 70% ice cold ethanol, dried and dissolved in a volume of RNase free water sufficient to achieve an RNA concentration of 300 ng/µl. At this point half of a volume of 5M solution of LiCl was added and the tube was left overnight on ice. After centrifugation and rinsing as before, the pure RNA was dissolved in 10 µl of RNase free water and stored at – 20°C. The RNA purity was determined with the nanodrop ND-1000 Spectrophotometer at the moment of the quantification, by considering the ratio of A_{260}/A_{280} for estimating peptides contamination and A_{260}/A_{230} for polysaccharides contamination. Satisfactory values for these two parameters were $1.8 \leq (A_{260}/A_{280}) \geq 2.0$ and $2.0 \leq (A_{260}/A_{230}) \geq 3.0$. When needed the precipitation steps were repeated. In order to further verify the RNA quality, a solution volume of 1µl was mixed with 5 µl formaldehyde loading buffer and incubated 5 minutes at 60°C for denaturing the RNA. The resulting solution was loaded on 1% agarose gel and separated by electrophoresis. In this way it is possible to detect the RNA quality by observing the resolution of 2 major bands corresponding to 28S and 18S ribosomal RNAs.

2.2.18 cDNA synthesis

First strand cDNA was synthesized using the Superscript™ II Reverse Transcriptase from Invitrogen (Karlsruhe, Germany). 3µg of total RNA was primed with 2.2 µl of Oligo(dT) 500 µg/µl and the protocol from the manufacturer was followed.

2.2.19 PCR conditions

The general reaction mix used for PCR was:

PCR buffer 10X including MgCl2	1µl
dNTPs 2 mM	1µl
Primer mix (forward + reverse) 10 µM	1µl
Taq DNA Polymerase	0.06 µl (0.3 U)
Template (10 to 100 ng/µl)	1µl
Distilled water	to 10 µl

Depending on the purpose of the PCR different enzymes were used. For the most common amplification reactions, like those for the polymorphic markers, the Taq DNA polymerase from Roche (Mannheim, Germany) was used. For Retro Transcription PCR: Taq DNA Polymerase from Qiagen (Hilden, Germany) For the amplification of templates of 4 to 6 Kb length: Expand Long Template Taq polymerase from Roche (Mannheim, Germany). For cloning of cDNA templates: AccuPrime™ *Pfx* DNA Polymerase from Invitrogen (Karlsruhe, Germany). In this last case the reaction conditions were adapted according to the instructions.

The amplification was performed using a Labcycler Basic from SensoQuest GmbH (Göttingen, Germany) or a Biozym multicycler PTC 240 from Biorad (Hercules, USA). The general reaction conditions were:

2 minutes	**94°C**	
20 seconds	**94°C**	**18,22 or 35 cycles**
30 seconds	**50-64°C**	
40 seconds	**72 or 68°C**	
10 minutes	**72 or 68°C**	
Stand by	**15°C**	

2.2.20 Molecular cloning techniques

Most of the constructs were prepared exploiting the Gateway® technology (Invitrogen, Karlsruhe, Germany) for direct cloning through a BP reaction of PCR products into pDNR207 Donor vector. For this purpose special primers were designed. The vectors were generally tested by restriction analysis and sequencing, also for verifying the absence of PCR induced mutations. Subsequently, through an LR reaction with the destination vectors containing a gateway cassette, the final constructs were obtained. Further checking with PCR and partial sequencing were used to ensure their correctness.

For cloning of specific PCR products the Zero Blunt® TOPO® PCR Cloning Kit from Invitrogen (Karlsruhe, Germany) was used, following the manufacturer's instructions.

2.2.20.1 Cloning of *SUA* genomic fragment into pGreen 0229

The bacterial artificial chromosome (BAC) including the *SUA* (At3G54230) genomic region of a Landsberg BIBAC2 genomic library (from the ABRC stock center, Chang et al., 2002) was identified with 2 PCR generated probes of about 900 bp length complementary to the genomic regions upstream and downstream of the *SUA* gene. These probes were labeled with the Megaprime Labeling Kit from Amersham Biosciences (England) and used for hybridization of the BAC library filters.

25 μg of the selected BAC clone was extracted from a large *E. coli* culture and digested with two enzymes, BstBI and AvrII, which cut within the open reading frame of the gene located 5′of *SUA* and in the intergenic region after the *SUA* 3′UTR.

At the same time 5μg of the binary vector pGreen 0229 were digested at the polylinker site with ClaI and SpeI.

Since these enzymes require different working conditions, the DNA fragments were precipitated after the first digestion step by adding Sodium Acetate 3 M (0.1 volumes) and pure ethanol (2.5 volumes), precipitated at – 80°C for half an hour, rinsed with 70% ethanol and resuspended.

The mixture of fragments obtained from the second digestion reaction of the BAC were separated on a 0.8% agarose gel, the band corresponding to the predicted size (9987 bp) was excised from the gel and purified. The same was done for the fragments derived from the digestion of pGreen 0229 (4157 bp), selecting the band of the linearized vector. The purified fragments (respectively 90 ng and 16 ng), having cohesive complementary ends, were ligated overnight at 16°C with the T4 DNA Ligase (Invitrogen), and transformation into *E. coli* followed. The pGreen 0229 BAC *SUA* clone was identified by colony PCR using the primer combination: pG0229f-824r.

Table 2.2.1: Constructs created during this work

Construct	Function	Transgenics selection
pAM-PAT 35S:*cSUA_GFP*	In Planta overexpression of the *SUA* cDNA fused in 3′ with *GFP*	BASTA
pBAT B *pSUA:cSUA_GFP*	Vector for the in planta expression of *cSUA_GFP* controlled by the native (-2711 bp) promoter	BASTA
pGreen 0229 BAC *SUA*	Construct for complementation of *sua-1*, carrying the *SUA* genomic region subcloned from a Landsberg BAC	BASTA
pGWB3 *pSUA:GUS*	Construct for in vivo studies of *SUA* promoter (-2711) controlling the reporter gene *GUS*	Kanamicyn + hygromycin
pLeela 35S:*cSUA*	overexpression of *SUA* cDNA genes in Planta	BASTA
pET-32	Inducible overexpression of His-tagged SUA protein in *E. coli*	Ampicillin

2.2.21 Bacteria Transformation

E. coli or *A. tumefaciens* competent cells were transformed with 100 ng plasmid, by heat shock or electroporation techniques (Hanahan, 1983; Dower et al., 1988). Transformant colonies were selected on solid LB or YEB media containing antibiotics and then grown overnight in a small volume of liquid culture. 1 µl of this cell suspension was used as template for PCR checking of the constructs.

2.2.22 Plant transformation

For every plant transformation experiment, 50 plants were grown in 10 (9 x 9 cm) pots in long day conditions. After approximately 3 weeks of growth the emerging flower buds were clipped as soon as they appeared, allowing the development of many secondary bolts. 7 to 10 days later, when the new stalks were flowering, the plants were transformed with the selected Agrobacteria strains, following the Floral Dip method (Clough and Bent, 1998). Agrobacterium was grown in liquid culture of YEB containing specific selection

antibiotics during three days, first in 5 ml than in 50 ml and finally in 500 ml culture, in shaking incubators at 28°C, until the optical density of the culture reached 0.8 OD_{600}. At this point the cells were precipitated at 4000 rpm for 15 minutes at 4°C and then resuspended into 400 ml 5% sucrose solution containing 0.03% silwet L70. This suspension was used for dipping the plants stalks for about 15 seconds, the plants were then left in a closed, dark container overnight, before returning to the greenhouse.

2.2.23 Transgenic plant selection

In order to select the transgenic plants carrying the BASTA resistance gene, plants were grown for 7-10 days on soil and sprayed with a 200 mg/l solution of the herbicide Glufosinat (BASTA, Höchst, Germany). In the case of transgenic plants resistant to Hygromicyn or Kanamicyn, the seeds were sterilized and sown on plates with half strength Murashige Skoog agar containing antibiotics (Kanamicyn: 50 mg/l, Hygromicyn: 25 mg/l). Two weeks after germination the resistant seedlings were transferred to soil.

2.2.24 GUS staining

All tissues from plants carrying the *SUA* promoter_*GUS* fusion transgene (from pGWB3 *pSUA:GUS*) were assayed for the GUS activity, according to Sessions et al., 1999. The plant material was submerged into the GUS Staining Buffer into large vials, and then vacuum was applied for 4 hours for ensuring optimal penetration of the Buffer. Incubation at 37°C overnight followed. The tissues were washed several times with pure ethanol to remove the chlorophyll.

2.2.25 Transient transformation of *Nicotiana benthamiana* leaves

The Agrobacterium strain carrying the construct of choice (pAM-PAT *35S_cSUA_GFP*) was grown in 5ml liquid YEB culture with selective antibiotics, until the OD_{600} was about 1. The bacterial cells were then collected by centrifuging at 5000 rpm for 10 minutes at 4°C and resuspended in 3 ml Induction Medium. The cell suspension was left for 2 hours at room temperature in the dark and then loaded into a needless 5 ml syringe. The fourth and fifth leave of young *Nicotiana benthamiana* plants were pierced once with a tip, and

injected with the Agrobacteria suspension from the lower side, opposing pressure with the finger on the other side. After two and three days the infiltrated leaves were dissected for microscopy preparations. For the confocal laser scanning microscopy a TCS SP2 AOBS from Leica (Wetzlar, Germany) was used. GFP was excited with 488 nm laser light, emission fluorescence was detected between 505 and 530 nm.

2.2.26 Protein expression in *E. Coli* and purification

The cDNA of *SUA*, excluding the stop codon, was cloned into pET32 vector, that includes sequences for his-tagging the insert in 5`. This construct was transformed into the *E. coli* strain BL21 (DE3) from Novagen (San Diego, USA).

The selected strain was grown overnight in 10 ml LB containing 100 µM Ampicillin and 150 µM Isopropyl-1-thio-ß-D-galactopyranoside (IPTG) in a shaker incubator at 20°C. The cells were collected through centrifugation at 4000 rpm for 15 minutes at 4°C. The pellet was resuspended in 2ml of a lysis/binding solution containing Lysozime 0.2 mg/ml and incubated on ice for one hour. Sarkosyl and Triton x-100 were added to reach a concentration of 0.2% (w/v and v/v, respectively). The cells in suspension were then destroyed with a UP50H sonicator (Dr. Hielscher, Teltow, Germany), repeating short pulses of 80% amplitude, until the suspension became clear. After centrifugation (14000 rpm, 4°C, 15 minutes) the supernatant was applied to the Ni^{++} column of the HisTrap FF crude Kit (GE Healthcare, München, Germany) and the protocol proposed by the manufacturer was followed.

2.2.27 Protein extraction from dry seeds

Two protocols were used for extracting total seed proteins. For immunoblotting 20 mg of dry seeds were weighed and ground in liquid nitrogen. 200µl of Protein Extraction Buffer (A) plus 50 µl DTT 1 mM were added to resuspend the ground seeds. The suspension was incubated at 4°C for 30 minutes and vortexed three times in between. The samples were then centrifuged twice for 10 minutes at 14000 rpm, at 4°C, transferring the supernatant into new tubes each time. The total protein extract obtained with this protocol was frozen at –20°C.

For the proteomic assay using 2D gels, another extraction method was used: 50 mg of seeds were ground in liquid nitrogen and resuspended with the Protein Extraction Buffer (B), completed with DTT 20 mM, protease inhibitors, DNase and RNase.
The cocktail was agitated at 4°C for one hour and centrifugated for removal of seed debris. The supernatant was treated with one volume of Protein Precipitation Solution and let overnight at -20°C. Two step of centrifugation and washing with Acetone (plus DTT 0.14 % w/v) followed. Finally the pellet was washed with absolute ethanol, dried and newly dissolved into the extraction buffer.

2.2.28 2D Gels

About 150 µg of proteins from the extracts were absorbed on 18 cm long acrylamide strips presenting a 3-10 non linear pH gradient (Bjellqvist et al., 1982). The isoelectricfocusing of the proteins resulted by applying 3500 V tension for 8 hours. The separation on the second dimension was obtained by positioning the strips on the top of large SDS-PAGEs (acrylamide 10%, piperazine diacrylamide 0.33%) and applying a 110 V tension for 14 hours. After separation the gels were stained with the silver nitrate technique (Rabilloud, 1999) and dried on a frame between 2 cellophane sheets.

2.2.29 SDS-PAGE

100 µg proteins mixed with enough Sample Buffer were incubated at 100°C for 4 minutes and loaded on 8% SDS-PAGE (Laemmli, 1970). Electrophoresis was performed by applying 40 mA current during 50 to 80 minutes. Then the gel was stained with Bio-Safe™ Comassie G-250 stain from Biorad (Hercules, USA).

2.2.30 Western Blot

After separation on SDS-PAGE the proteins were blotted on a PVDF membrane (Millipore, USA) through semi-dry electrotransfer for 75 minutes at 2.8 mA/cm2. The immunological reactions of primary and secondary antibodies with the immobilized target proteins were done in 10-15 ml of Blocking Buffer.

3 Results

3.1 Characterization and fine mapping of seed longevity natural modifiers in *abi3-5* and *lec1-3* mutant backgrounds

3.1.1 Selection of modifier lines and mapping populations

Arabidopsis is adapted to a wide range of environments across the northern hemisphere, which is reflected in genetic variation between different accessions. This variation also concerns seed physiology aspects directly connected to the complex trait of longevity. In recent years quantitative trait loci (QTL) mapping approaches using recombinant inbred lines populations was used to identify QTLs involved in seed longevity (Bentsink et al., 2000; Clerkx et al., 2004b) based on Controlled Deterioration Test (CDT). Another approach to study the genetics of seed longevity is to combine natural variation with mutants that are hypersensitive to desiccation damage such as *abi3-5* and *lec1-3* mutants. Seeds of these mutants completely lose viability within a few weeks after harvest, whereas wild-type seeds can still germinate after several years. ABI3 and LEC1 are transcription factors that are abundant during seed development and which are responsible for the activation of maturation specific genes that lead to reserve accumulation and to the establishment of desiccation tolerance. Emile Clerkx and colleagues at the Wageningen University selected introgression lines with enhanced seed longevity from crosses of *abi3-5* and *lec1-3*, in Landsberg *erecta* (L*er*) background, with 20 accessions originating from different geographic locations. F2 progenies, which were homozygous respectively for *abi3-5* or *lec1-3* were tested for seed longevity. The genotype of these lines contain 50% L*er* DNA on average. The lines with the highest viability after a short period of storage were backcrossed twice to the L*er* parent. During these crosses the homozygous *abi3-5* and *lec1-3* mutations were maintained and the proportion of exotic genomes was reduced. The resulting lines were genotyped using the bulked segregant analysis (Michelmore et al. 1991) and the approximate map position of each modifier was determined (Clerkx et al., 2004c; Figure 3.1.1). Modifiers of *abi3-5* from the Eilenburg (*abi*/Eil), Warschau (*abi*/Wa) and Seis am Schlern (*abi*/Sei) accessions, and two modifers of *lec1-3* both originated from the Shahdara accession (*lec*/Sha1 and *lec*/Sha2) were selected for further study.

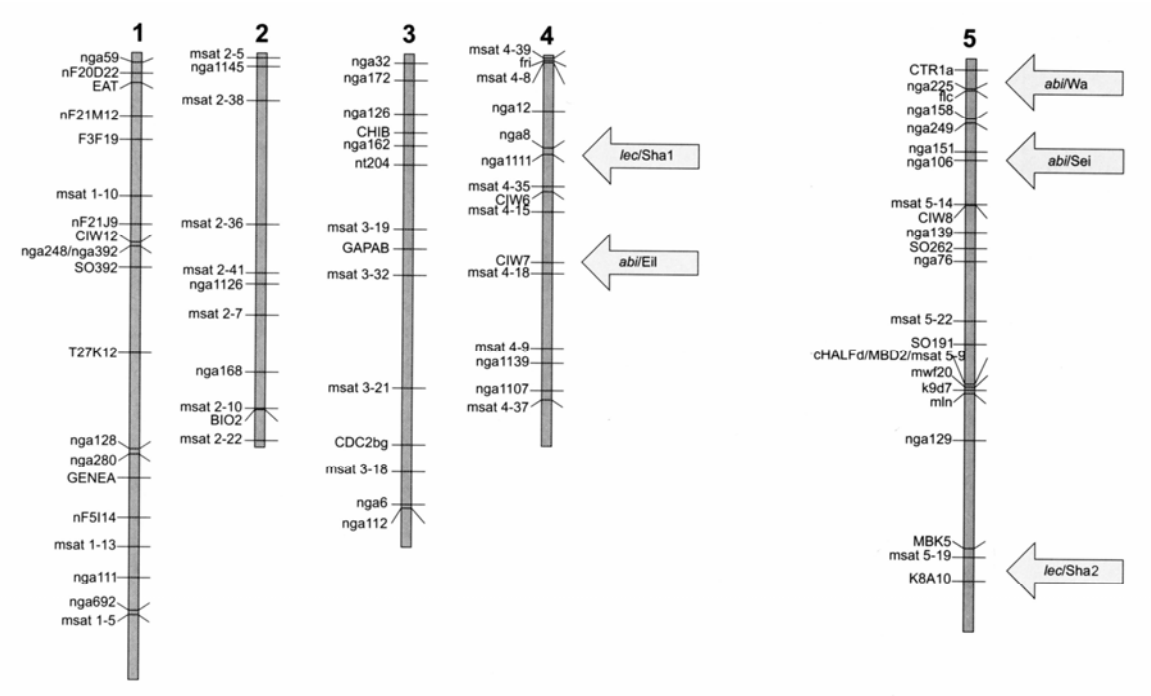


Figure 3.1.1: Genetic map positions of the *abi*/Eil, *abi*/Sei, *abi*/Wa, *lec*/Sha1 and *lec*/Sha2 modifiers as previously determined by Clerkx (2004c).

The use of newly designed SSLP polymorphic markers indicated the presence of multiple introgressions from the exotic accessions in the modifier lines, which is expected after two backcrosses. In order to reduce the possible interference of additional seed longevity modifiers, more backcrosses were made. The lines *lec*/Sha1 and *lec*/Sha2 were backcrossed four more times with *lec1-3*, *abi*/Sei was backrossed three times and *abi*/Eil and *abi*/Wa once with *abi3-5*, and re-selected according to the procedure described in figure 2.1. After every backcross, F2 populations homozygous for *abi3-5* or *lec1-3*, and segregating for the modifier's introgressions were grown for mapping experiments.

Every mapping population was genotyped with a few polymorphic markers located in the chromosomal regions containing the modifier (Figure 3.1.1) and in regions that were not homozygous for L*er* based on the first genetic survey. In this marker assisted selection most of the introgressions that were not significantly linked to the longevity trait could be excluded. Seed batches of F3 lines were tested for their viability immediately after harvest and after seed storage. This analysis led to the confirmation of three modifiers: *abi*/Sei, *lec*/Sha1 and *lec*/Sha2.

For the modifiers *abi*/Eil and *abi*/Wa a significant linkage between the introgressed regions and the improvement of seed longevity could not be confirmed. The distribution of

longevity of seed batches from F2 plants, homozygous for *abi3-5*, could not be grouped into distinct phenotypic classes (Figure 3.1.2 as an example). This means that seeds from F2 plants with the *abi*/Eil or *abi*/Wa introgression did not show improved seed longevity compared to seeds from the *abi3-5* mutant in L*er* background.

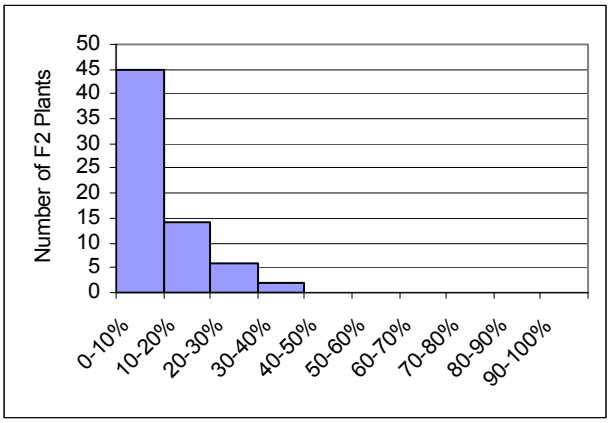


Figure 3.1.2: Frequency distribution of viability in the progeny (BC2 F3) of seeds from a mapping population homozygous for *abi3-5* and segregating for the Eilenburg introgression on chromosome 4. The germination test was performed after one month of storage; none of the F2 plants showed increased seed viability.

In the populations segregating for *abi*/Sei, *lec*/Sha1 and *lec*/Sha2 F3 lines with increased longevity were found (see Figures 3.1.3, 3.1.4 and 3.1.5). However, discrete phenotypic classes could not be distinguished. This is probably due to the quantitative character of seed longevity, which is strongly influenced by environmental factors acting during seed development and storage.

For final phenotyping, the F2 plants were grouped based on the germinability of their F3 seeds after one month of storage. At this time point seeds from *abi3-5* and *lec1-3* in L*er* background showed less than 20% germination. The longevity phenotype of the F3 seed batches was divided into a class with germination between 0 and 40% and a class with germination between 40 and 100%.

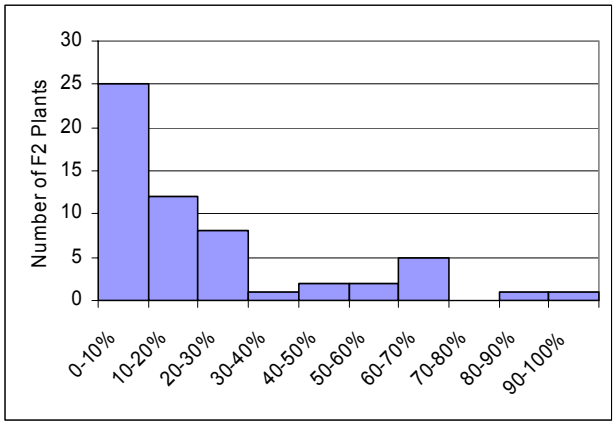


Figure 3.1.3: Frequency distribution of viability in the progeny (BC4 F3) seeds from an *abi*/Sei mapping population homozygous for *abi3-5* and segregating for the Seis am Schlern introgression on chromosome 5. The germination test was performed after one month of storage.

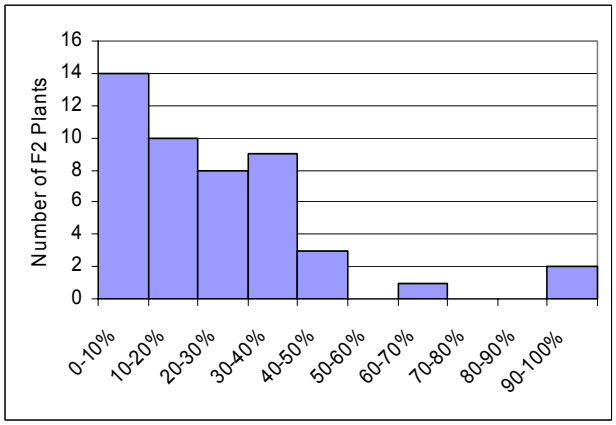


Figure 3.1.4: Frequency distribution of viability in the progeny (BC5 F3) seeds from a *lec*/Sha1 mapping population homozygous for *lec1-3* and segregating for the Shahdara introgression on chromosome 4. The germination test was performed after one month of storage.

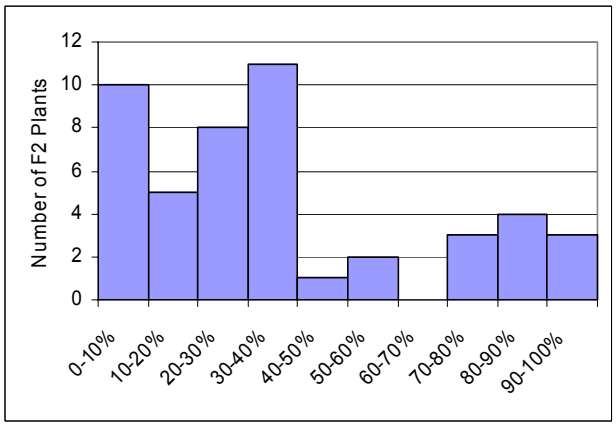


Figure 3.1.5: Frequency distribution of viability in the progeny (BC5 F3) seeds from a *lec*/Sha2 mapping population homozygous for *lec1-3* and segregating for the Shahdara introgression on chromosome 5. The germination test was performed after one month of storage.

The observed distribution of longevity phenotypes in the segregating populations of *abi*/Sei, *lec*/Sha1 and *lec*/Sha2 suggested that seed longevity was influenced by modifiers from the Sei and Sha accessions, although a clear Mendelian distribution could not be observed. A population segregating for both Shahdara introgressions in *lec1-3* background was also tested and showed that Sha1 and Sha2 modifiers additively increase the longevity trait. About 44% (7/16) of this population was homozygous for at least one modifier (Figure 3.1.6).

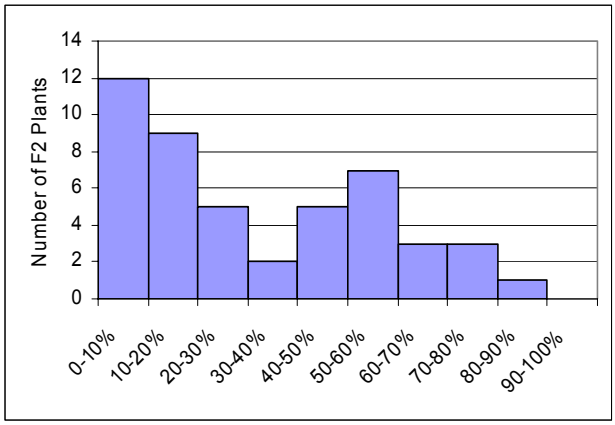


Figure 3.1.6: Frequency distribution of viability in the progeny (BC4 F3) seeds from a *lec*/Sha1/Sha2 mapping population homozygous for *lec1-3* and segregating for the Shahdara introgressions on chromosome 4 and 5. The germination test was performed after one month of storage.

3.1.2 Detailed genotyping of the modifier lines

To further decrease Sei and Sha DNA in the modifier lines, additional backcrosses with *abi3-5* or *lec1-3* in L*er* background were made and the lines with highest longevity were selected. A more accurate genetic mapping was performed by integrating additional polymorphic markers information and more dense genetic maps were drawn. The Sei introgression with the *abi*/Sei modifier was confirmed in the top of chromosome 5, while the Sha chromosomal segments defining the *lec*/Sha1 and the *lec*/Sha2 modifiers were mapped in the top of chromosome 4 and the bottom of chromosome 5, respectively (Figures 3.1.7, 3.1.8 and 3.1.9).

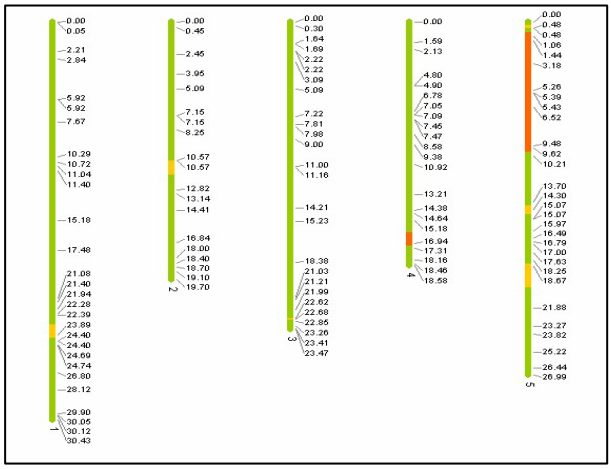


Figure 3.1.7: Genetic map of the *abi*/Sei modifier line. The Sei introgression (orange) is located between 0.48 Mb and 10.21 Mb on Chromosome 5. The map is defined by 34 SSLP and 82 SNP polymorphic markers; the corresponding map positions are indicated (Mb). The L*er* genotype is represented in green and the heterozygous regions in yellow.

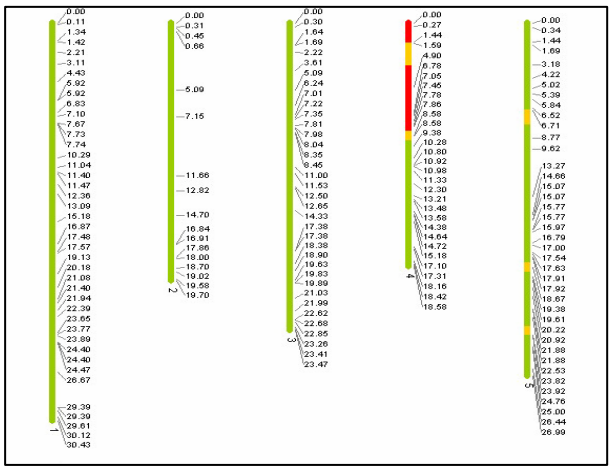


Figure 3.1.8: Genetic map of the *lec*/Sha1 modifier line. The Sha introgression (red) is located between 1.59 Mb and 8.58 Mb on Chromosome 4. The map is defined by 49 SSLP and 120 SNP polymorphic markers; the corresponding map positions are indicated (Mb). The L*er* genotype is represented in green and the heterozygous regions in yellow.

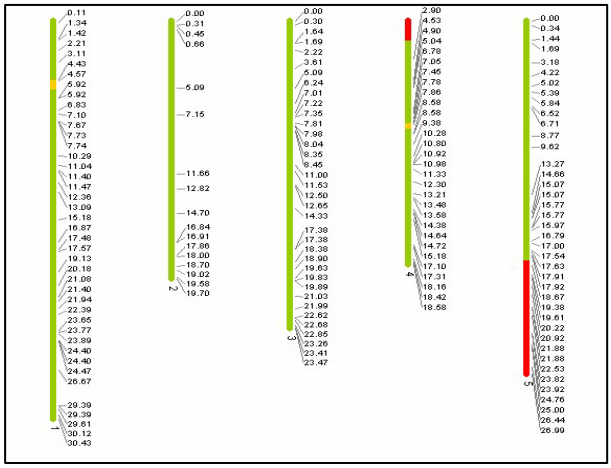


Figure 3.1.9: Genetic map of the *lec*/Sha2 modifier line. The Sha introgression (red) is located between 17.92 Mb and 26.99 Mb on Chromosome 5. The map is defined by 49 SSLP and 121 SNP polymorphic markers; the corresponding map positions are indicated (Mb). The L*er* genotype is represented in green and the heterozygous regions in yellow.

3.1.3 Confirmation of the position of the modifiers

The presence of a modifier introgression did not always correlate with improved longevity in the segregating populations. The significance of the increased longevity by the modifiers was analyzed with a one way Analysis of Variance (ANOVA) statistical test of the segregating populations. Eight markers located within the modifiers regions were treated separately. For each marker, the F2 individuals were divided into a group that was homozygous for L*er* for that marker and a group that was homozygous for Sha or Sei. Heterozygous individuals were excluded from the analysis. The two groups were compared for their average seed longevity. The ANOVA test showed a strong correlation between the presence of the homozygous modifier alleles and improved seed longevity, for every locus tested. For each modifier region, some loci were linked with higher significance to longevity than others (Table 3.1.1, 3.1.2 and 3.1.3); the results of this test indicated that *abi*/Sei maps close to the marker F2P16, lec/Sha1 is most likely located between T17A2 and T26M18, whereas lec/Sha2 is most closely linked to MDF20. These

results definitely confirmed the modifiers as enhancers of seed longevity and provided new information about their map position.

Table 3.1.1: One way ANOVA test on the phenotypic and genotypic data of 110 F2 plants from a population segregating for the Sei introgression and homozygous for *abi3-5*. The significance that the Sei allele is correlated with improved longevity is shown for every marker. The physical position of each locus is indicated within brackets.

***abi*/Sei (markers Chromosome 5)**		**Mean Square**	**F**	**Significance**
MED24 (1.05 Mb)	between groups	0.112	4.964	<0.028
	Within groups	0.023		
MTG13 (5.46 Mb)	between groups	0.358	18.413	<0.000
	Within groups	0.019		
F2P16 (9.47 Mb)	between groups	0.504	25.272	<0.000
	Within groups	0.02		

Table 3.1.2: One way ANOVA test on the phenotypic and genotypic data of 72 F2 plants from a population segregating for the Sha1 introgression and homozygous for *lec1-3*. The significance that the Sha allele is correlated with improved longevity is shown for every marker. The physical position of each locus is indicated within brackets.

***lec*/Sha1 (markers Chromosome 4)**		**Mean Square**	**F**	**Significance**
T17A2 (4.9 Mb)	between groups	0.529	16.583	<0.000
	within groups	0.032		
T26M18 (7.1 Mb)	between groups	0.357	17.463	<0.000
	within groups	0.02		

Table 3.1.3: One way ANOVA test on the phenotypic and genotypic data of 69 F2 plants from a population segregating for the Sha2 introgression and homozygous for *lec1-3*. The significance that the Sha allele is correlated with improved longevity is shown for every marker. The map position of each locus is indicated within brackets.

***lec*/Sha2 (markers Chromosome 5)**		**Mean Square**	**F**	**Significance**
MPL12 (18.8 Mb)	between groups	0.61	8.923	<0.004
	within groups	0.068		
MDF20 (22.5 Mb)	between groups	0.574	9.413	<0.003
	within groups	0.061		
K8A10 (26.4 Mb)	between groups	0.143	1.938	<0.169
	within groups	0.074		

3.1.4 Longevity phenotypes of the modifier lines

The three modifier lines described in paragraph 3.1.2, were grown together with *abi3-5* and *lec1-3* and wild-type L*er*. After harvest the seeds were stored at room temperature in a closed, dark container. Germination of the seeds was tested during a period of several weeks in a time course experiment (Figure 3.1.10).

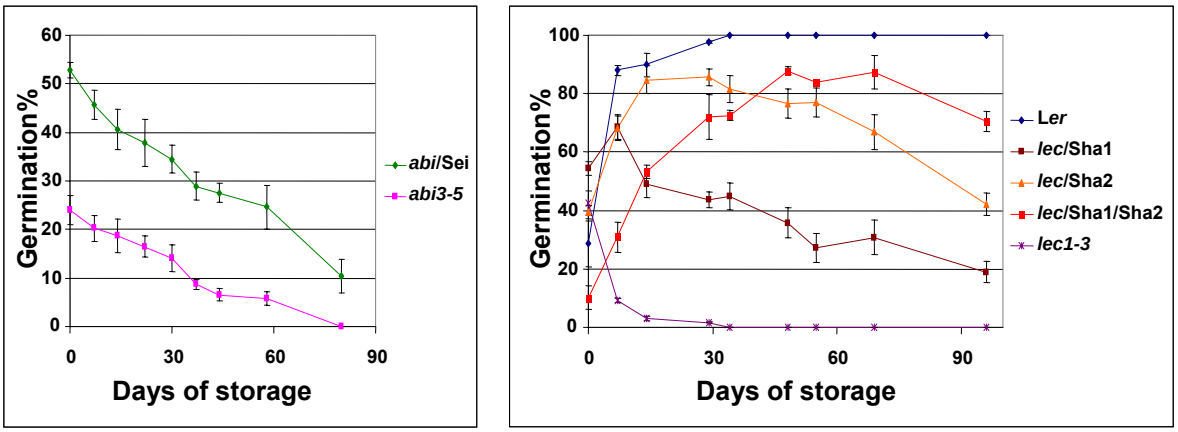


Figure 3.1.10: Germination percentage during seed storage of *abi*/Sei modifier and *abi3-5* mutant (left), and of L*er*, *lec*/Sha1, *lec*/Sha2 and *lec*/Sha1/Sha2 and *lec1-3* mutant (right). Data points show the average of five biological replicates. Standard errors are indicated.

The *abi*/Sei modifier line showed a higher germination than *abi3-5* at all times after harvest, but the rate by which its seed viability decreases did not differ. This might indicate that the beneficial effects of the *abi*/Sei modifier are restricted to the pre-harvest phase.

The seeds from the modifier lines *lec*/Sha1 and *lec*/Sha2 showed a much higher germination than the *lec1-3* parent, except immediately after harvest. This is probably caused by dormancy of the *lec*/Sha1 and *lec*/Sha2 modifier seeds because in these experiments no stratification treatment was provided. This suggests a substantial change in the seed physiology in the *lec*/Sha lines because the *lec1-3* mutation in its original L*er* background lacks seed dormancy (Raz et al., 2001).

The line with both introgressions from Shahdara (*lec*/Sha1/Sha2) showed the highest longevity and strongest dormancy phenotypes, indicating that the *lec*/Sha1 and *lec*/Sha2 lines carry independent modifiers with additive effects for both traits.

3.1.5 Sha modifiers increase seed weight of *lec1-3* mutant

Seed weight in Arabidopsis is not correlated with longevity, but seedlings derived from heavier seeds have a better chance to establish into soil after germination (Krannitz et al., 1991). Substantial genetic variation has been found in Arabidopsis for this trait (Alonso-Blanco et al., 1999). Previous studies showed that in *abi3* mutants the accumulation of storage proteins is reduced (Nambara et al., 1992; Parcy et al., 1994), but the accumulation of sucrose is increased (Ooms et al., 1993). Therefore, the seed weight of *abi3-5* does not vary significantly from wild-type L*er*. The *lec1-3* mutation causes a reduced accumulation of storage compounds, particularly proteins and oils causing a flattened and shrivelled appearance of the seeds (Meinke et al., 1994) and a reduced weight. The modifier line *abi*/Sei produced seeds with a similar weight as the wild-type and *abi3-5* controls. In contrast, seeds from the two *lec*/Sha modifier lines were significantly heavier than *lec1-3* mutant seeds (Figure 3.1.11). Notably, the *lec*/Sha1/Sha2 seeds were even heavier than the L*er* control ones. Seeds from the Sha accession were the heaviest measured, suggesting the involvement of alleles with quantitative effects on seed weight that could co-locate with the Sha longevity modifiers.

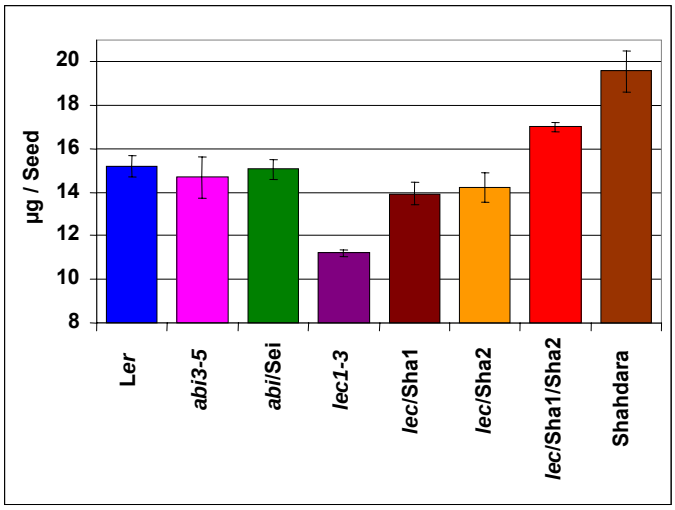


Figure 3.1.11: Average seed weight of longevity modifiers and controls. For each measurement batches of a precise number of seeds comprised between 400 and 500 were equilibrated during one week at 11.3% relative humidity and then weighed.
The values represent the means of five biological replicates, standard errors are indicated.

3.1.6 Seed proteomics with two-dimensional gels

The proteomic profile of seeds from the modifiers, mutants and wild-type was determined using the 2D gel technique in collaboration with Dr. Löic Rajjou from the group of Prof. Marc Jullien at the Laboratoire de Biologie des Semences (UMR204 INRA/AgroParisTech in Paris). Proteomic profiling can provide robust information about the biological

functions affected in the seeds under study by revealing their protein composition (Pandey and Mann, 2000). About 10% of the Arabidopsis seed proteome can be visualized by this method because only the most abundant proteins are detectable. The produced data was used for a qualitative description and comparison of the modifiers and their controls. Bulked seeds from ten sibling plants per line were used for the protein extraction and four technical replicates of the 2D gels were produced for each sample extract.

3.1.6.1 Comparative study of the wild-type, *abi3-5* and *lec1-3* seed proteomes

Proteins were extracted from freshly harvested seeds of L*er*, *abi3-5* and *lec1-3* and separated on 2D gels. The location of protein spots that showed a difference in intensity between mutant and wild-type was compared with the existing Arabidopsis seed proteome maps. (Gallardo et al., 2002; Rajjou et al., 2004, 2006; Job et al., 2005; http://www.seed-proteome.com).

Both mutants exhibited a substantial reduction of seed storage proteins (SSPs) compared to the L*er* protein profile. This was particularly evident for the clusters of protein spots representing the 12S globulins (Figure 3.1.12), which is in accordance with previous findings on the physiology of these mutants (Nambara et al., 1992; Parcy et al., 1994; Meinke et al., 1994). The *lec1-3* and *abi3-5* mutant profiles also showed a higher abundance of proteins that typically increase in germinating seeds.

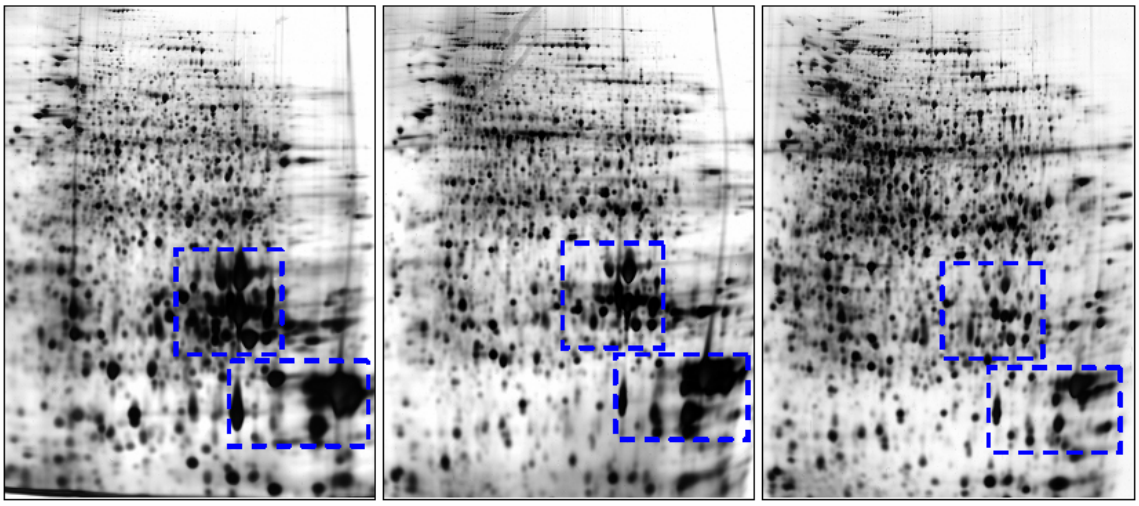


Figure 3.1.12: 2D-proteome profiles of L*er* dry mature seeds (left), *abi3-5* mutant seeds (middle) and *lec1-3* mutant seeds (right). The blue, dashed rectangles highlight the clusters of spots which mostly represent the storage globulins.

By visually comparing the 2D proteome profiles of L*er* with *abi3-5* or *lec1-3*, all the spots occupying the same gel positions but having different intensity were marked. About 100 of these spots could be linked to proteins previously identified and positioned on the proteomic reference maps. The proteins that showed an increased or decreased abundance in *abi3-5* or *lec1-3* mutant seeds compared to the wild-type (Figures 3.1.13 and 3.1.14) are listed in the Tables 3.1.4 and 3.1.5 (Appendix). Apart from a reduced abundance of seed storage globulins and their precursors (see spot 18 as an example), *abi3-5* or *lec1-3* seeds also lacked proteins necessary for surviving desiccation, such as two late embryo abundant proteins (LEAs) corresponding to At2G42560 and At3G17520 (spot 23 and 35) and heat shock proteins (HSPs) like the product of At5G12030 (spot 16 and 53). At the same time proteins related to seed germination, such as the enzymes involved in the glyoxylate cycle and photosynthesis (rubisco precursors, spots 10 and 11) were increased, providing molecular evidence of the non-dormant phenotype of the two mutants. The enzymes related to stress response and cell detoxification like the glutathione-dependent dehydroascorbate reductase (spot 7) were also increased in *abi3-5* and *lec1-3* and were probably induced by the seed deterioration processes of oxidative nature. Many of the highlighted spots corresponded to yet unidentified proteins.

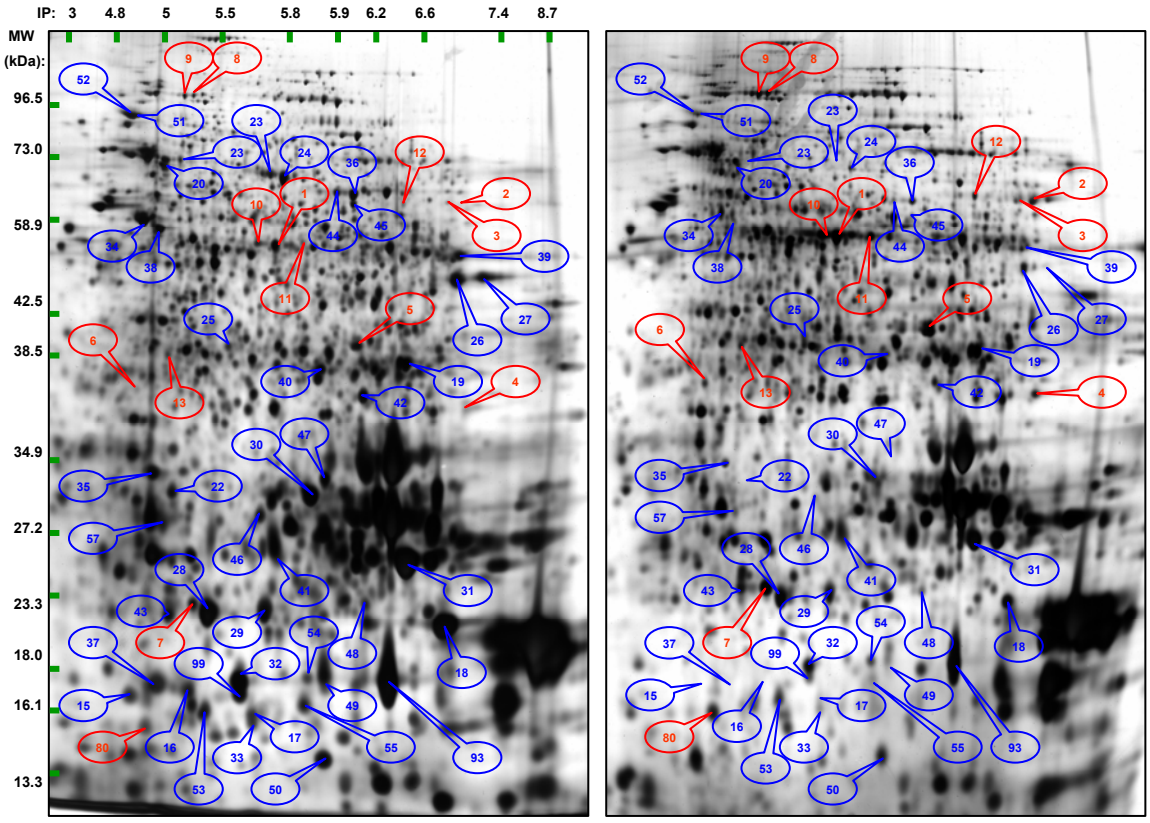


Figure 3.1.13: Comparative analysis of L*er* (left) and *abi3-5* (right) seed proteomes. The blue balloons indicate proteins with decreased abundance in *abi3-5*, the red baloons indicate proteins with increased abundance. See Table 3.1.4 in Appendix for the identity of the proteins. Molecular weight (MW, vertical) and isoelectric point (IP, horizontal) are shown.

Seeds from the *abi3-5* mutant were specifically characterized by a lower abundance of desiccation tolerance related proteins like the LEAs (spots 34 and 38) and HSP70 (spot 20). Increased quantities of enzymes involved in the catabolism of lipids were also detected, in particular isocitrate lyase (spot 12, see Table 3.1.4 in Appendix and Figure 3.1.13).

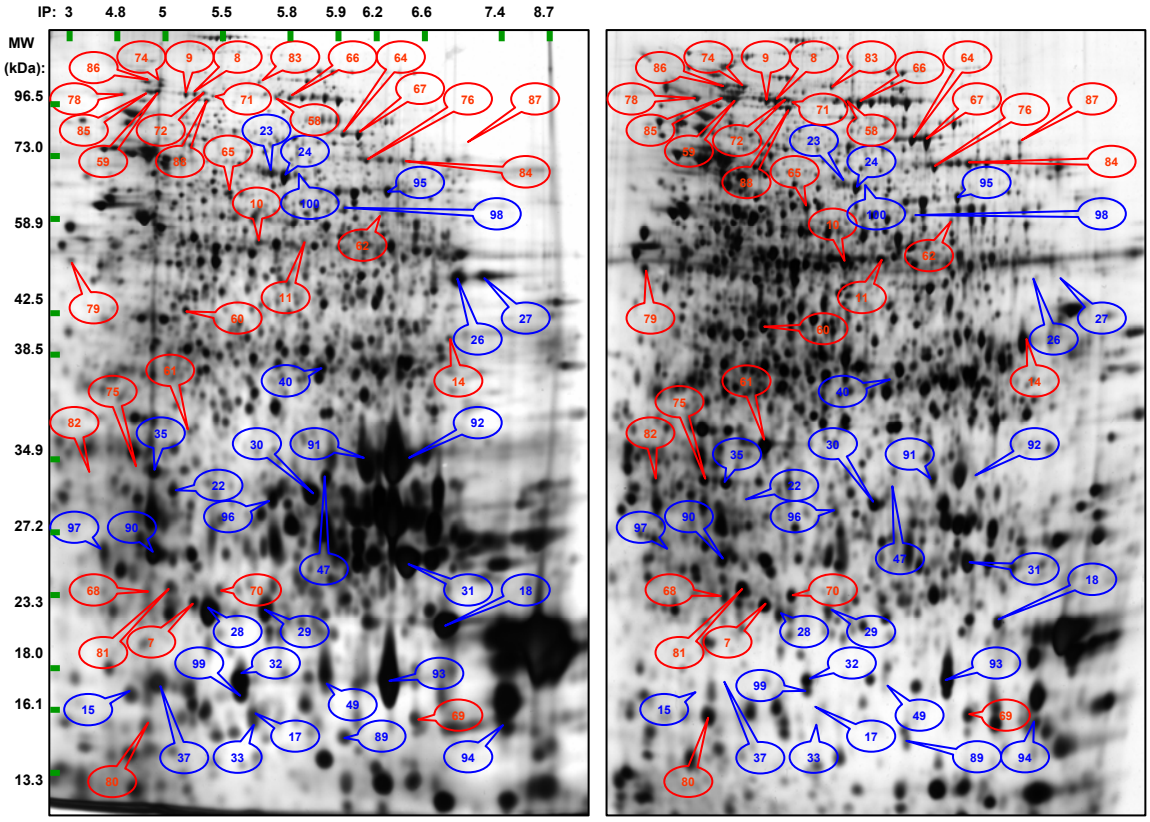


Figure 3.1.14: comparative analysis of L*er* (left) and *lec1-3* (right) seed proteomes. The blue balloons indicate proteins with decreased in the mutant, the red ones indicate proteins with increased abundance. See table 3.1.5 in Appendix for the identity of the proteins. Molecular weight (MW, vertical) and isoelectric point (IP, horizontal) are shown.

The *lec1-3* mutant seeds showed a more dramatic decrease of 12 S globulins and of LEAs (Figure 3.1.14). Many germination and energy metabolism related proteins like the cell division cycle protein CDC48 (spots 59, 74 and 85) and the enzyme phosphoenolpyruvate carboxykinase (spot 84) were strongly increased. Jasmonate (JA) hormone inducible proteins (spots 61, 62 and 65) were also more abundant in *lec1-3* than in L*er* (see Table 3.1.5 in Appendix). The synthesis of JA is direct consequence of membrane damage (Creelman and Mullet, 1997).

3.1.6.2 Proteome analysis of the modifier lines

The seed proteome profile from the *abi*/Sei modifier line was almost identical to that of the *abi3-5* mutant. A significant difference in the storage protein abundance could not be detected. A single spot appeared to be characteristic for the modifier line (Figure 3.1.15, blue and red circles). This protein might be a Sei accession specific isoform of a yet unidentified protein, present in the *abi3-5* and in the L*er* profiles, which focused at a gel

position corresponding to the same molecular weight, but at a lower isoelectric point. Such isoform originates from genetic polymorphism and might not be a determinant of seed longevity.

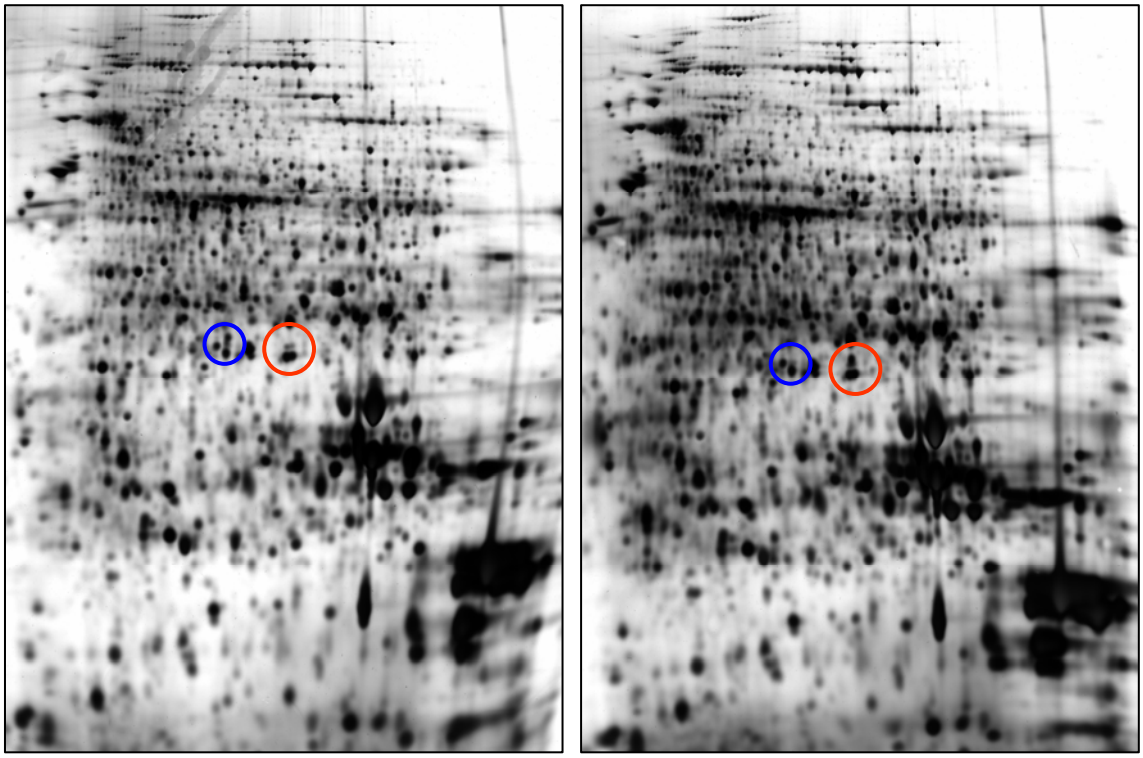


Figure 3.1.15: Comparative analysis of the *abi3-5* (left) and *abi*/Sei (right) seed proteomes. The circles highlight two specific protein spots that differ between the two protein samples.

The proteomic profiles of the *lec*/Sha modifier lines were very different from that of the *lec1-3* mutant. Most of the storage globulins, HSPs and LEAs were restored close to wild-type levels (Figure 3.1.16). The abundance of 12S storage proteins was higher in *lec*/Sha2 than in *lec*/Sha1 and the line containing both Shahdara (Sha) modifiers showed the highest levels (Figure 3.1.16, brown and blue windows). In addition, many protein spots specific to the *lec1-3* proteome, such as jasmonate inducible proteins, and, in lesser extent, germination related proteins, such as cell cycle proteins and the enzyme phosphoenolpyruvate carboxykinase, were maintained higher than in L*er* (See the red arrow in Figure 3.1.16 as an example).

Every proteome profile showed a specific protein signature (Figure 3.1.16, red and green windows). The abundance of storage proteins correlated with longevity and dormancy phenotypes.

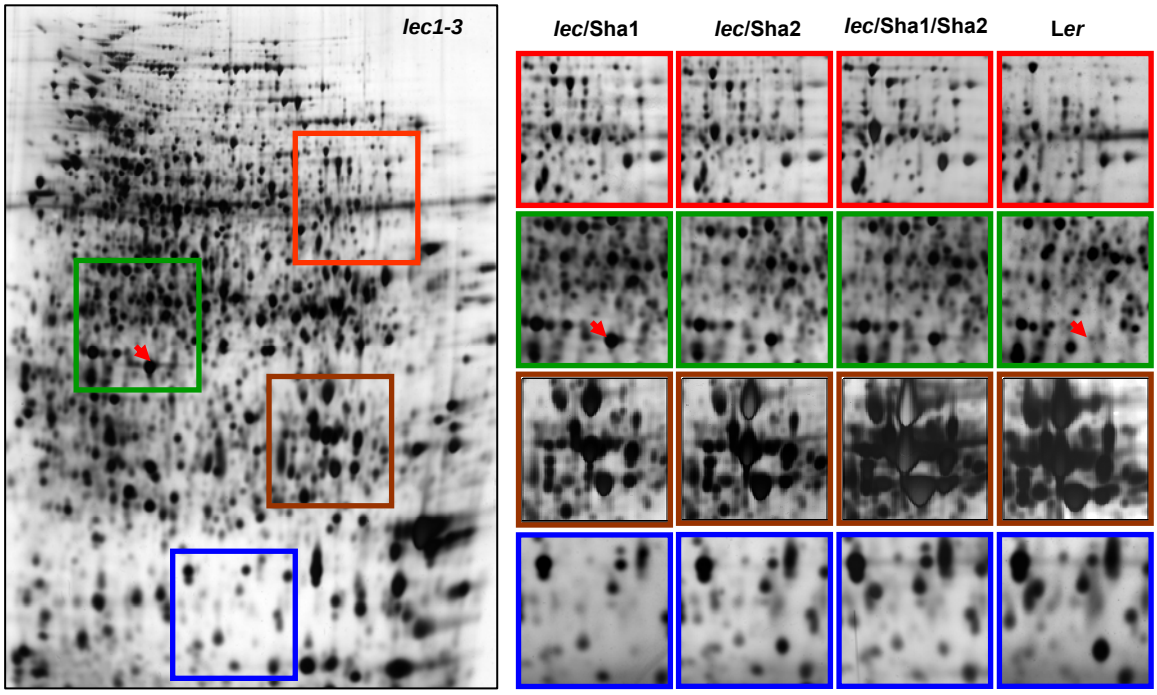


Figure 3.1.16: Comparative analysis between *lec1-3*, the Sha modifier lines and L*er* seed proteome profiles. The red arrows indicate the spot 61, corresponding to a Jasmonate inducible protein.

The spot 96, equivalent to the enzyme aspartic proteinase, was increased in the *lec*/Sha modifier proteomes in a similar way as the SSPs were (Figure 3.1.17, blue arrow). The expression of the aspartic proteinase gene is preferentially induced by the FUS3 transcription factor, which in turn is regulated by LEC1 (Kagaya et al., 2005). The spot 96 in the *abi3-5* proteome profile had an intensity similar to the wild-type, consistent with the presence of LEC1 and FUS3.

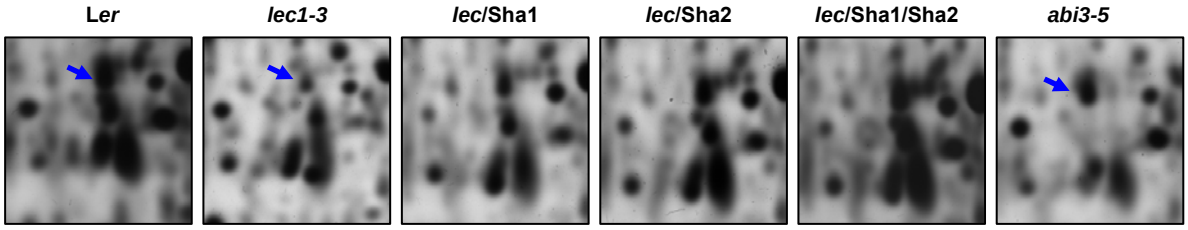


Figure 3.1.17: Comparative analysis between L*er*, *lec1-3*, the Sha modifier lines and *abi3-5* seed proteome profiles. The blue arrows indicate the spot 96, corresponding to the aspartic proteinase enzyme.

3.1.7 Controlled deterioration of the parental accessions and Sha near isogenic lines

To determine if the parental accessions from which the modifiers were derived display differences in longevity, a Controlled Deterioration Test (CDT) using three years old wild-type seeds was performed. High relative humidity (85%) and temperature (37°C) were provided to create an environment in which all seeds quickly deteriorated. Seeds from the Sha accession showed the highest resistance to this artificial aging treatment, while Sei did not differ from L*er* (Figure 3.1.18). This result confirms that the natural variation between L*er* and Sha accessions can be exploited for identifying QTLs of seed longevity (Clerkx et al., 2004b).

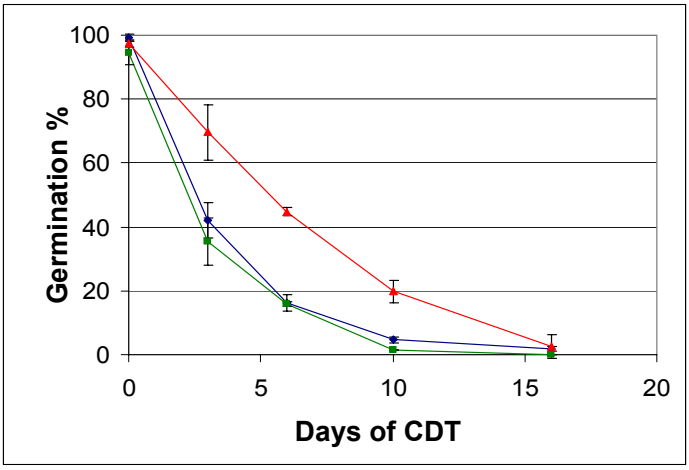


Figure 3.1.18: Germination of seeds after the controlled deterioration treatment. Three years old seeds from Ler (blue line), Sha (red) and Sei (green) were tested for germination after 3, 6, 10 and 16 days of incubation. Data points represent the average of 3 biological replicates, standard errors are indicated.

Subsequently, dry mature seeds from four near isogenic lines (NILs) of Sha in L*er* background were tested in a CDT. These NILs have overlapping Sha introgressions in the region at the bottom of chromosome 5, from 21 to 26.8 Mb (see Table 2.1: LShetb1, LShetb2, LShetb3 and 5b.17), which contains the strongest *lec1-3* modifier, *lec*/Sha2. This test was performed to assess if the effects of modifier on longevity in a *lec1-3* background could also be detected in a wild-type background. All NILs performed more similarly to L*er* than to Sha, but the NIL with the whole Sha region (5b.17), and LShetb3, after six days of artificial aging proved more resistant than L*er* and the other NILs (Figure 3.1.19), while LShetb1 was the least resistant. This result indicates that two QTLs for seed longevity with minor effects map in the region from 21 to 26.8 Mb at the bottom of chromosome 5. The Sha introgression in LShetb1 decreases the trait, while Sha in LShetb3 increases it. The NIL 5b.17 has a phenotype consistent with the effects of both QTLs.

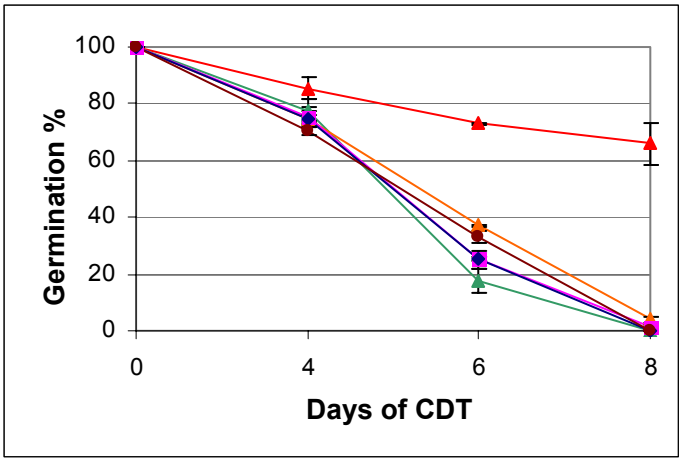


Figure 3.1.19: Germination of seeds after the controlled deterioration treatment. Seeds from L*er* (blue line), Sha (red), LShetb1 (green), LShetb2 (pink), LShetb3 (orange) and 5b.17 were tested for germination after 4, 6 and 8 days of incubation. Data points represent the average of 6 biological replicates, standard errors are indicated.

3.2 Characterization and cloning of *SUPPRESSOR OF abi3-5* (*SUA*)

3.2.1 Previous work on the isolation and mapping of *sua* mutants

The ABI3 protein is a transcription factor necessary for the activation of genes implicated in seed maturation and the establishment of desiccation tolerance (Parcy et al., 1994; Kotak et al., 2007). ABI3 is a transducer of the abscisic acid (ABA) hormone signal and a regulator of the transition between embryo maturation and early seedling development (Nambara et al., 1995). Arabidopsis plants with mutations in the *ABI3* gene are disturbed in the processes that prepare the embryo for the dry state that ensures its survival after dispersal. The *abi3-5* mutant allele has a single nucleotide deletion that causes a frame shift and the formation of a premature stop codon upstream of the functional B2 and B3 domains, necessary for the interaction of ABI3 with other transcription factors and for the *cis* activation of seed specific promoters (Bies-Ethève et al., 1999). The *abi3-5* mutant seeds display major changes in gene expression that result in a reduced accumulation of storage proteins, like 12s globulins, and of proteins with a protective role during desiccation such as heat shock proteins and late embryogenesis abundant proteins (see chapter 3.1.7.1). The *abi3-5* seeds are non dormant due to a decrease in ABA sensitivity during germination, and green because chlorophyll is not degraded during seed maturation (Ooms et al., 1993). These changes in *abi3-5* seeds lead to a strong reduction in longevity. Seeds remain viable only for a few weeks when they are stored at room temperature (RT). Because the *abi3-5* mutation affects many seed properties, a mutagenesis screen of *abi3-5* might yield enhancers or suppressors mutants involved in specific subsets of the maturation processes controlled by ABI3.

Emile Clerkx and colleagues in Wageningen University mutagenized *abi3-5 gl1 tt5-1* triple mutant seeds with gamma irradiation (Clerkx, 2004c). The *gl1* and *tt5-1* mutations were used as phenotypic markers on both sides of *ABI3* locus on chromosome three. These markers could be used to distinguish mutants that reverted the *abi3-5* phenotypes completely from wild-type contaminants. Selection for improved longevity in M3 seed batches yielded four suppressor mutants that survived storage longer than *abi3-5 gl1 tt5-1* control seeds. These lines were quadruple mutants that carried novel suppressor mutations of *abi3-5* (therefore called *sua*) and were named *sua1*, *sua2*, *sua3* and *sua4*

respectively. The *sua* mutants were backcrossed to L*er*, which verified that they were all recessive mutations. They were also crossed among each other, which showed that the mutations were not allelic to each other. Finally, F2 mapping populations were generated by crossing the mutants with the *glabra* mutant in Columbia (Col) background. Approximate map positions of the *sua* mutants were estimated with the Bulked Segregant Analysis method (Michelmore et al., 1991; Figure 3.2.1). The work referred above was used as a starting point for the analysis of the suppressor mutants described in this thesis.

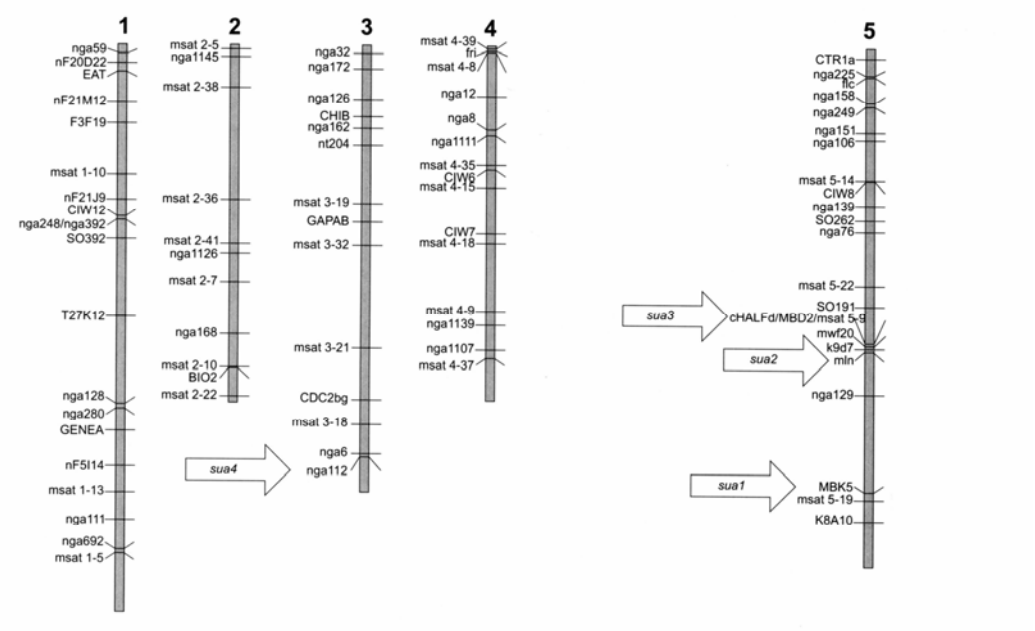


Figure 3.2.1: Genetic map with the positions of the *sua* mutants as previously determined by Clerkx (2004c). The map is based on 81 markers polymorphic between the L*er* and Col accessions.

3.2.2 Seed longevity of the *sua* mutants in different storage conditions

In an initial experiment the phenotypes of the four *sua* quadruple mutants were re-evaluated. Seed batches from suppressor and control lines were harvested at maturity and stored under different conditions in order to obtain an accurate description of the longevity phenotypes. Each seed batch, bulked from six sister plants, was divided in aliquots of 150-200 seeds. From each mutant, ten aliquots were stored different conditions: the -80°C freezer, the Samenlager (the seed storage room at the Max Planck Institute for Plant Breeding Research with 30% relative humidity (RH) and 5°C), four exicators with respectively 23, 42, 69 and 94% RH at RT (18-22°C). The seeds were withdrawn periodically from each storage environment and tested for germination (Figure 3.2.2).

Over a period of eleven weeks there was no significant loss of germinability for seeds stored in the -80°C freezer. Interestingly, also the dormancy alleviation, which is normally observed for L*er* seeds stored at RT was severely reduced at – 80°C. Most biochemical reactions are limited in frozen state because the molecular mobility is strongly reduced. The release of dormancy is a process that requires metabolic changes, particularly in ABA catabolism (Gubler et al., 2005).

Seeds of the *sua* mutants and the *abi3-5* control did not display dormancy phenotypes, therefore the observed decrease in germinability during storage would be due to seed death. Storage at 23% and 42% RH and RT was most informative for the phenotyping of the *sua* mutants because seed deterioration occurred gradually in these conditions. Seeds derived from *sua1* and *sua2* showed high germination immediately after harvest and a similar rate of deterioration as monogenic *abi3-5* mutant seeds (Figure 3.2.2). The *sua3* seeds germinated close to 100% immediately after harvest, but in some of the treatments they deteriorated faster than any other seed batch. In the specific experimental conditions that were tested, the *sua3* mutation did not behave as an enhancer of seed longevity, in contrast to what was observed by Clerkx (2004c). The *sua4* seeds germinated always at a great pace and *sua4* was the only mutant that could be confirmed as a suppressor of the *abi3-5* longevity phenotype. The viability of *sua4* seeds decreased only in the most drastic storage conditions, which also affected the L*er* control seeds (see the 69% RH graph and 94% RH graph in Figure 3.2.2).

The *sua* mutant seeds gradually deteriorate and their viability is not expected to increase at any time because they are not dormant. However, fluctuations in the germination were often observed. This could be due to variation in uncontrolled environmental factors, such as the O_2 or CO_2 concentration in the incubator, which could influence the rate of germination. The O_2 pressure was shown to be correlated with germination (Corbineau and Côme, 1995) because germinating seeds use oxygen for their intense catabolic metabolism. The oscillations in germinability affected most of the genotypes in a similar way (see the -80°C graph and 23% RH graph in Figure 3.2.2). These observations did not affect the overall evaluation of the analyzed mutants.

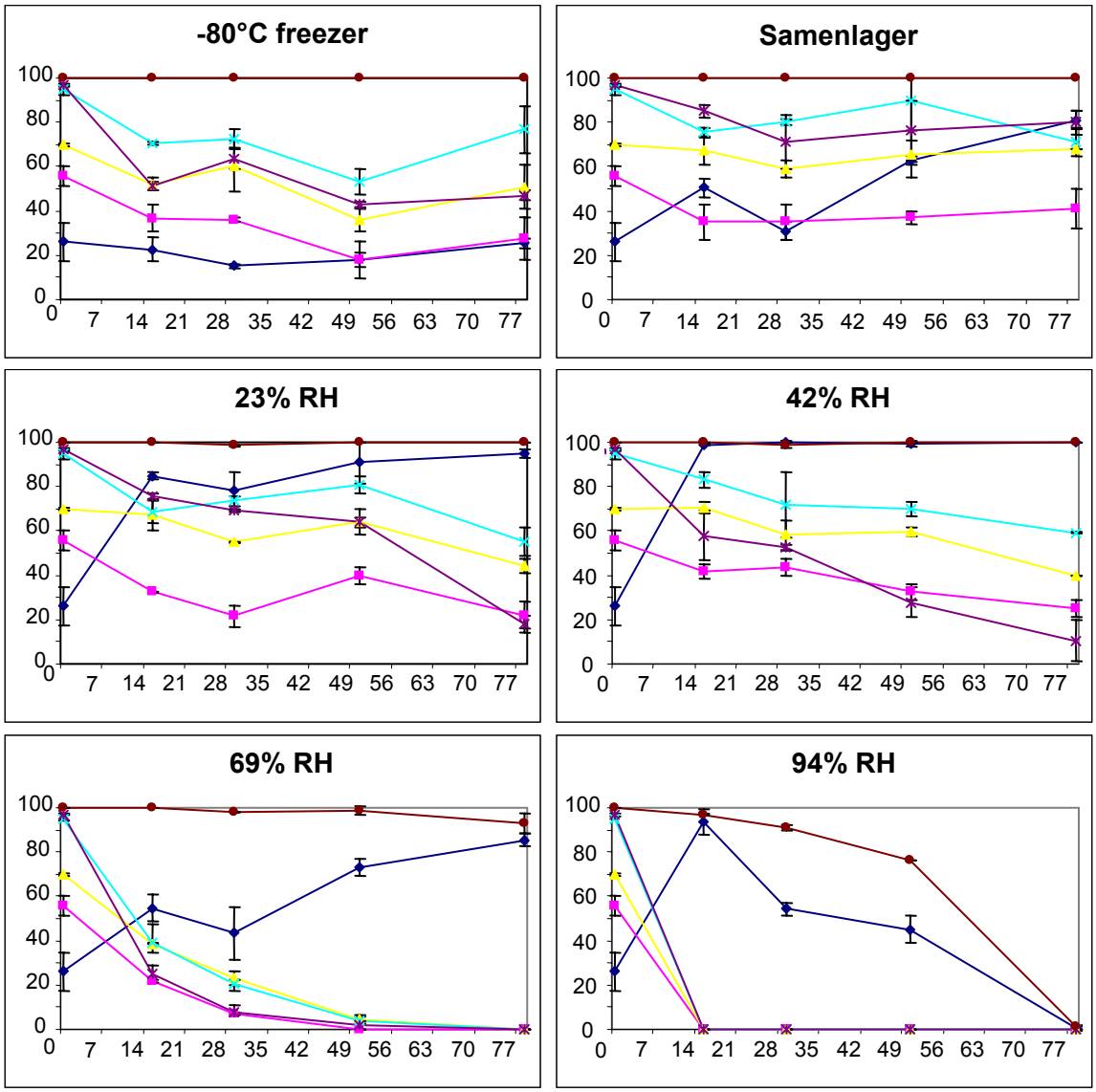


Figure 3.2.2: Germination percentage of L*er* (blue line), *sua1* (yellow), *sua2* (light blue), *sua3* (violet), *sua4* (brown) and *abi3-5* (pink) immediately after harvest and after storage for 16, 30, 51 and 80 days in different conditions. The four *sua* mutants also include *abi3-5 tt5-1* and *gl1* mutations. Data points represent the average of two replicates of six bulked plants from each line. The bars represent standard errors.

3.2.3 Chlorophyll content in seeds

The *sua* mutations also affected the green seed color that is characteristic for *abi3-5* mutants. In particular *sua4 abi3-5 tt5-1 gl1* was characterized by a reduced green color of the seeds (Figure 3.2.3). The pigments responsible for the green color in *abi3-5* seeds are the chlorophylls, which are degraded during seed maturation in wild-type seeds (Nambara et al., 1995; Parcy et al., 1997).

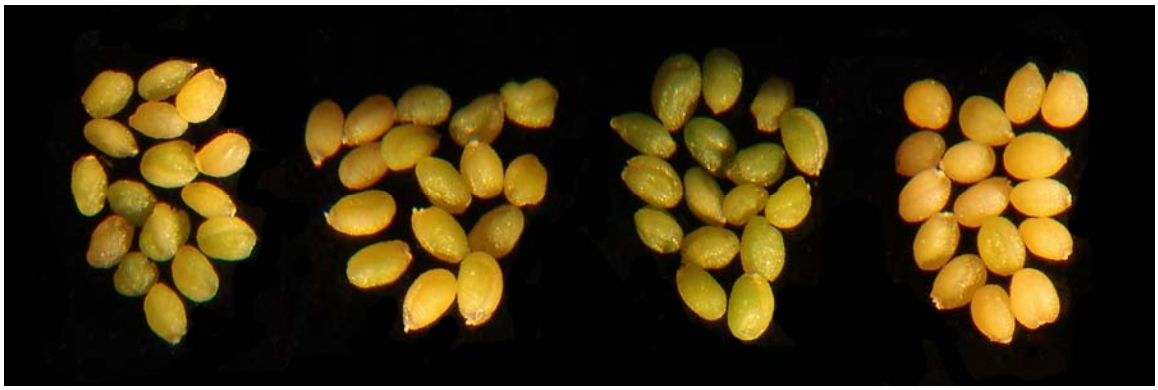


Figure 3.2.3**:** From left to right: seeds from *sua1*, *sua2*, *sua3* and *sua4* lines.

Previous studies in *Brassica oleracea* have shown that high amounts of chlorophyll in the seeds correlate with reduced longevity (Jalink et al., 1998). Therefore, the chlorophyll content in the *sua* mutant seeds was quantified. Total chlorophylls were extracted from the seed batches used for the germination tests shown in the paragraph 3.2.2, and quantified as described in paragraph 2.2.11. A very low concentration of chlorophyll was detected in L*er* seeds, but *abi3-5* seeds contained about 0.5 mg chlorophyll per gram of seeds. The *sua3* mutant contained a similar amount of chlorophyll as *abi3-5*, while *sua1* and *sua2* had between 0.3 and 0.4 mg chlorophyll per gram of seeds. In *sua4* the chlorophyll content was almost 0.1 mg per gram and only slightly higher than in L*er* (Figure 3.2.4).

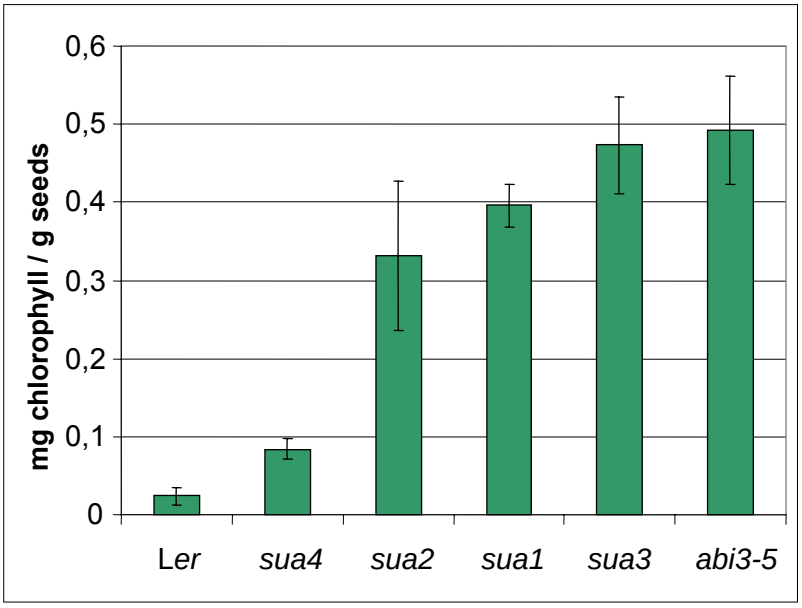


Figure 3.2.4: Chlorophyll content in L*er*, *sua1-4* and *abi3-5*. Bars represent the average of two measurements on seed pools from six sister plants. Standard deviations are indicated.

3.2.4 Measurement of the oxidative damage in seeds

Seeds stored in aerobic conditions will be damaged by oxygen radicals. The oxygen gas can penetrate the integuments and the cellular membranes and triggers the formation of active oxygen species (Inzé and Van Montagu, 1995; Bailly, 2004). Damage to membranes is a direct consequence of the oxygen radical attack on the phospholipids layers. DNA and proteins also accumulate damage, up to a level that prevents germination. A direct correlation exists between aging and the oxidation of cellular macromolecules (Stadtman, 2001). The oxidative damage on seeds can be estimated with the FOX method (see paragraph 2.2.10), that quantifies the level of lipid peroxides. This method analyzes the membrane phospholipids and the oil reserves that constitute 30-40% of the dry weight of wild-type Arabidopsis seeds (Yonghua et al., 2006). The level of lipid peroxides was found to increase with the age of the seeds (data not shown). This is in agreement with the accumulation of seed tissue damage due to the oxygen radical reactions that occur during storage. Seed batches from L*er*, *sua1*, *sua2*, *sua3*, *sua4* and *abi3-5* were tested. Seeds from *lec1-3* and the modifier lines *abi*/Sei and *lec*/Sha1/Sha2, described in chapter 3.1, were also included in the analysis. After one month of storage L*er* seeds contained the least peroxides. The genotypes *sua1*, *sua3*, *abi3-5* and *abi*/Sei had similar values of 90-110 μM hydrogen peroxide equivalents per mg of seeds, *sua2* had about 80 μM, whereas *sua4* contained only 55 μM, just 10 to 15 μM equivalents more than L*er* (Figure 3.2.5). In the seed batches characterized by the *abi3-5* mutation, a direct correlation between the peroxidation level and the concentration of chlorophyll was observed (see Figure 3.2.4, *abi*/Sei not tested), which suggests that residual pigments from the photosystems in the seeds could catalyze the formation of oxygen radicals. A strong correlation with the longevity phenotypes was found for L*er*, *sua1*, *sua2*, *sua3*, *sua4* and *abi3-5* (compare Figure 3.2.5 with Figure 3.2.2), and also for the *abi*/Sei modifier. For *lec1-3* and *lec*/Sha1/Sha2 the lipid peroxides were 52 and 62 μM hydrogen peroxide equivalents per mg of seeds respectively. These values might be affected by the anthocyanins that accumulate in *lec1* mutant embryos (Meinke et al., 1994), which have a strong antioxidant effects. Probably because of the anthocyanins, the seed longevity phenotypes did not correlate with lipid peroxidation levels in *lec1-3* mutants. The results here described indicate that, besides the chlorophyll and the lipid peroxide levels, there must be other factors that result in decreased longevity.

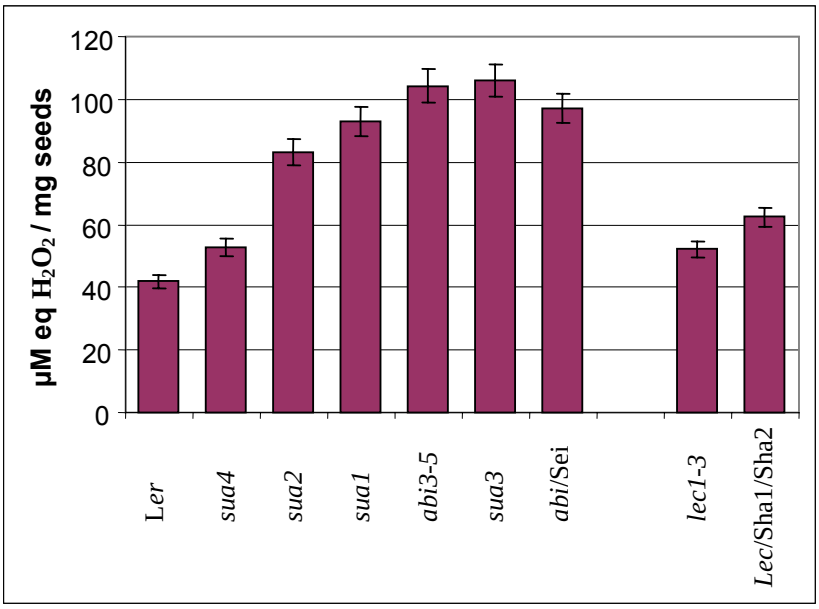


Figure 3.2.5: Lipid peroxidation levels expressed as µM hydrogen peroxide equivalents per mg of seeds.
Bars represent the average of two measurements on seed pools from six sister plants.
Standard deviations are indicated.

3.2.5 ABA sensitivity at germination

Seeds that contain the *abi3-5* mutation have an altered ABA response during imbibition. Wild-type seeds are almost completely unable to germinate in the presence of three µM ABA. In contrast, *abi3-5* seeds are completely insensitive to the inhibitory effects of ABA and can even germinate in the presence of 1 mM ABA. Analysis of the suppressor lines *sua1*, *sua2* and *sua3* showed that they were all insensitive to ABA, similarly to *abi3-5* (data not shown). The *sua4* seeds displayed a moderate sensitivity to ABA. The seeds were unable to germinate at concentrations higher than 20 µM, but could germinate at lower concentrations, which made them clearly distinguishable from both *abi3-5* and L*er* seeds (Figure 3.2.6). This feature was exploited for the identification of the *sua4* mutants within segregating populations homozygous for *abi3-5*. Germination tests on ABA solutions with concentration of 5 and 20 µM respectively allowed the unequivocal identification of the *sua4* mutants.

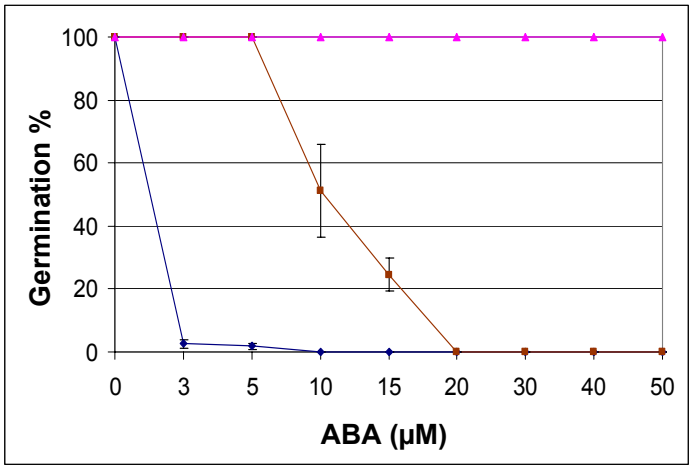


Figure 3.2.6: Abscisic acid sensitivity at germination for *abi3-5* (pink), *sua4 abi3-5 gl1 tt5-1* (brown) and L*er* (blue). The percentage of germination was calculated as the proportion of viable seeds that could develop into a seedling. Dead seeds were not considered. Data points are the average of five biological replicates, standard deviations are indicated.

3.2.6 Mapping of the *sua* mutants

The longevity phenotype of *sua3* could not be confirmed (see paragraph 3.2.2) and therefore this mutant was excluded from mapping experiments. The other *sua* mutants all contained a *sua* mutation in combination with *abi3-5*, *tt5-1* and *gl1*. These quadruple mutants were crossed with Cvi NILs (Keurentjes et al., 2007; Table 2.1.3), that contained Cvi introgressions in the regions were the *sua* mutations were previously mapped (Figure 3.2.1). This led to the creation of mapping populations with predominantly L*er* genetic backgrounds in which the natural variation between L*er* and Cvi would not interfere with the recognition of *sua* mutant phenotypes. The location of the *sua* mutants, obtained by Emile Clerkx (Figure 3.2.1), was used to select the NILs. The *sua1* mutant was crossed with LCN 5-8 and LCN 5-15, that covered the region from 8 to 25 Mb on chromosome 5; the *sua2* mutant was crossed with LCN 5-8, LCN 5-15 and LCN 5-19, that covered the region from 8 to 26.8 Mb on chromosome 5 and the *sua4* mutant was crossed with LCN 3-12 and LCN 3-16, that covered the region between 8 and 23.5 Mb on chromosome 3. F2 seeds from each population were germinated on ABA in order to select plants homozygous for *abi3-5*. Polymorphisms between L*er* and Cvi in the introgressed regions were used to map the *sua* mutants with molecular markers. F2 populations were genotyped with molecular markers and for the phenotyping seed batches from F2 plants were tested for germination one and four weeks after harvest. The location of *sua1* and *sua2* mutations, as determined by Clerkx (2004c), could not be confirmed. In the mapping of *sua2* for example, no linkage was found between germination after one week of storage and the markers on chromosome 5 (Figure 3.2.7 and 3.2.8). Possibly the original locations of these loci (Figure 3.2.1) were wrong or the longevity phenotypes were too weak and variable for a reliable scoring under the

conditions used in the present experiments. Therefore, mapping of *sua1* and *sua2* was not continued.

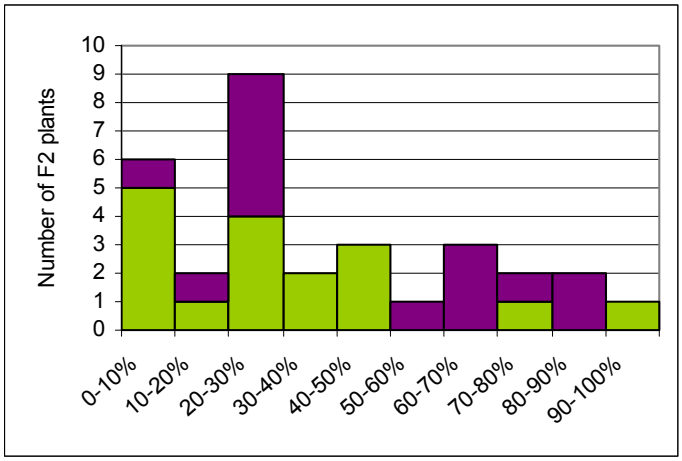


Figure 3.2.7: Germination percentage of 68 F2 plants from the cross between *sua2* and LCN 5-8 after one week of storage. Plants homozygous L*er* for nga139 (8.4 Mb, chromosome 5) are shown in green and plants homozygous Cvi for nga139 in purple. Heterozygous plants are not shown.

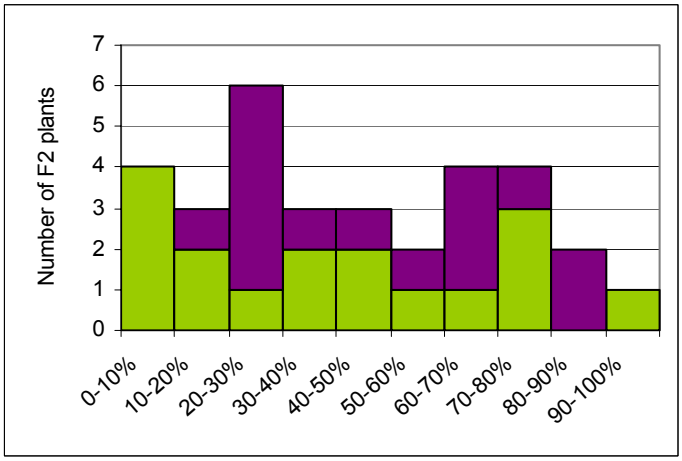


Figure 3.2.8: Germination percentage of 68 F2 plants from the cross between *sua2* and LCN 5-8 after one week of storage. Plants homozygous L*er* for nga129 (20.1 Mb, chromosome 5) are shown in green and plants homozygous Cvi for nga139 in purple. Heterozygous plants are not shown.

In all *sua1*, *sua2* and *sua4* mapping populations, a correlation between the *tt5-1* marker (20.44 Mb on chromosome 3) and improved germinability was observed (Figure 3.2.9). This indicates that the presence of the *tt5-1* mutation can influence the detection of seed longevity modifiers.

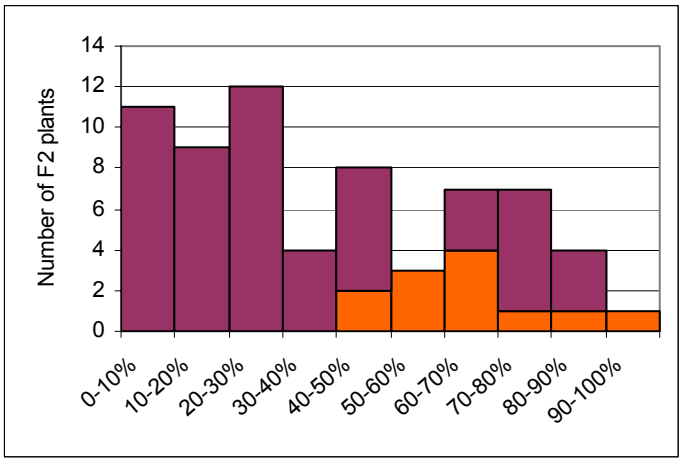


Figure 3.2.9: Germination percentage of 68 F2 plants from the cross between *sua2* and LCN 5-8 after one week of storage. Plants homozygous for *tt5-1* (20.44 Mb, chromosome 3) are shown in orange and plants that yielded seeds with normal testa (*tt5-1*/*TT5* or *TT5*/*TT5*) in purple.

Analysis of the segregating population from the cross between *sua4* and LCN 3-12 showed a clear correlation between improved seed longevity and the presence of the region containing the *sua4* suppressor mutants reported by Clerkx et al (2004c). From 168 F2 plants, 40 yielded seeds that germinated nearly 100% after 2 months of storage. This is consistent with a 3:1 segregation of a recessive suppressor mutant. Seeds from these plants were also characterized by an increased ABA sensitivity, compared to the *abi3-5* mutant. They could germinate well on 5 μM ABA solutions, but were unable to germinate on 20 μM ABA, which is a characteristic feature of the *sua4 abi3-5* double mutant seeds (Figure 3.2.6). These results showed that the fine mapping of the *sua4* mutant was feasible.

From the 40 homozygous *sua4* plants, 39 were also homozygous for *tt5-1*, which indicated a strong linkage between the two genes. Because the *tt5-1* mutant affected the germinability phenotypes in other *sua*/NIL Cvi populations (Figure 3.2.9), it was expected to influence the *sua4* phenotype as well.

The 168 F2 plants from the *sua4*/LCN 3-12 population were genotyped with six molecular markers and the *tt5-1* phenotypic marker, located in the region that contained the *sua4* mutation, between 16.3 and 20.44 Mb on chromosome 3. Two recombinants on both sides of *sua4* were identified; one with F8J2 (19.6 Mb) and one with *tt5-1* (20.44 Mb). Therefore, the location of *sua4* was narrowed down within a region of about 800 kb (Figure 3.2.10).

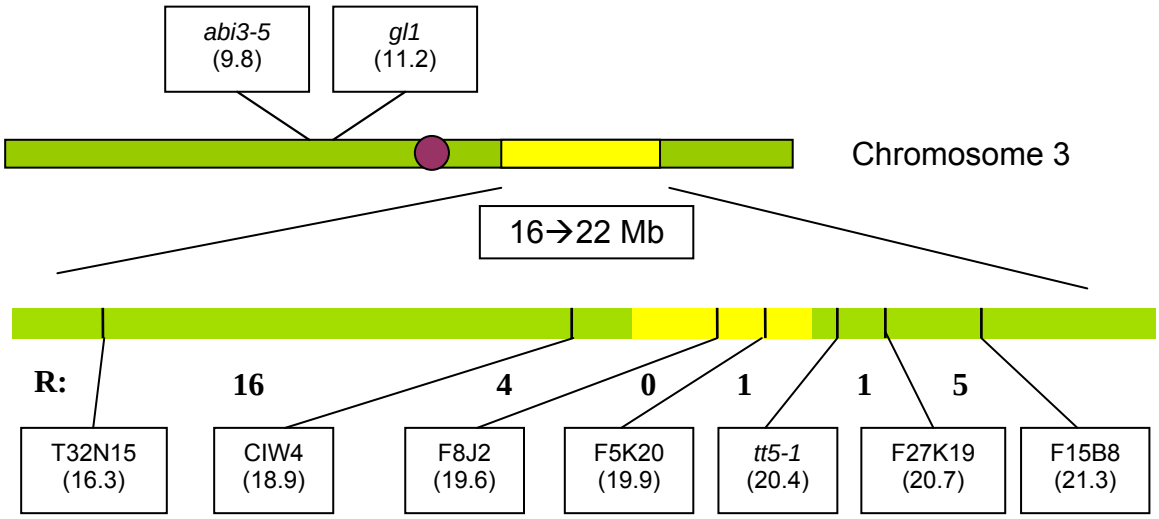


Figure 3.2.10: Rough mapping of the *sua4* mutation in the *sua4*/LCN 3-12 F2 population. The markers used for the mapping and their physical positions (Mb) are indicated in the boxes. The numbers in bold between the markers indicate the overall number of recombinants (**R**) identified within each interval among the 168 plants analyzed

3.2.7 *tt5-1* is an enhancer of seed germination

The mapping experiments described in paragraph 3.2.6 revealed that the *tt5-1* mutation influenced seed longevity, and could be epistatic to suppressor mutants with weak effects. This result is unexpected since the seed coat provides protection to the embryo (Haughn and Chaudhury, 2005) and natural aging of various Arabidopsis testa mutants, including *tt5-1*, showed a lower longevity compared to the wild-type (Debeaujon et al., 2000). To clarify this issue, a controlled deterioration test was set up for L*er* and *tt5-1* mutant seeds in L*er* background. The seeds were incubated in an exicator with 94% RH at RT for 80 days and samples were taken at different time points and tested for germination. After six weeks *tt5-1* mutant seeds germinated consistently better than L*er* seeds (Figure 3.2.11). This result is in contrast to what previously observed by Debeaujon et al (2000) and might indicate that the artificial deterioration at high relative humidity does not mimic ageing at room conditions. At high moisture content the increased seed metabolism reactions, such as mitochondrial respiration, might result in a type of damage that is different from the progressive and slow tissue deterioration that occurs naturally during long term dry storage.

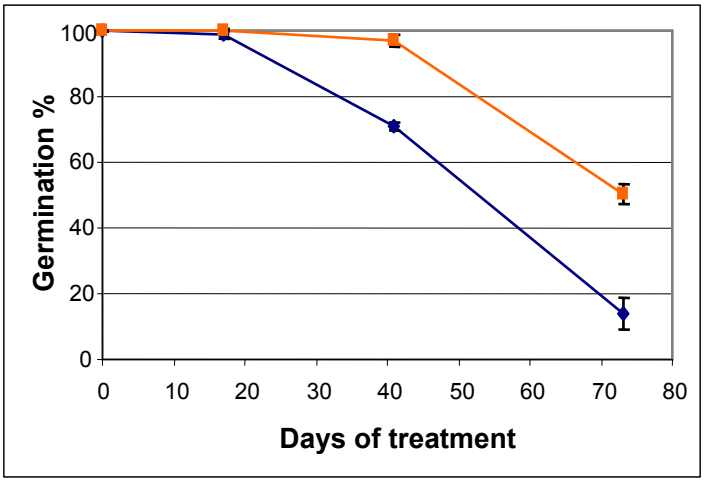


Figure 3.2.11: Germination percentage of L*er* (blue line) and *tt5-1* (orange line) seeds after storage at 94% RH. Data points are the average of two replicates of five bulked plants from each line, standard errors are indicated.

Because of the influence of *tt5-1* on longevity, the *sua4* mutant was backcrossed with L*er* to select *sua4* plants that were homozygous *TT5*. Among 96 F2 plants homozygous for *abi3-5* and *sua4*, two individuals without *tt5-1* phenotypes were selected for further backcrossing. Because the testa originates from maternal tissue, plants heterozygous for the *tt5-1* allele do not segregate for testa color in the progeny seeds. For this reason, the F3 generation had to be analyzed to select for homozygous *TT5* plants. During these backcrosses the *gl1* mutation was also crossed out.

3.2.8 Fine mapping of *sua4*

The *sua4 abi3-5* double mutant in a *TT5* and *GL1* background was crossed with the Col accession to generate new mapping populations. Because of the clear *sua4* phenotypes the effect of potential Col modifiers was expected not to hamper the identification of *sua4 abi3-5* double mutants. The advantage these new mapping populations was the availability of sequence polymorphism information between L*er* and Col (https://www.arabidopsis.org/cgi-bin/cereon/), which was exploited to generate molecular markers (Table 3.2.1 in Appendix). F2 seeds of the mapping populations were germinated on 5 µM ABA to select for *abi3-5* seedlings. The homozygous double mutants *sua4 abi3-5* were identified by their browner seeds, compared to *abi3-5* single mutants (see paragraph 3.2.3), which were unable to germinate on 20 µM ABA solutions (see paragraph 3.2.5). The progeny of F2 plants homozygous for *abi3-5* and heterozygous for L*er* and Col alleles between the markers CIW4 and *tt5-1* (Figure 3.2.10), were used for further fine-mapping of

sua4. The phenotype of every F3 plant was determined by the seed color. Brown seeds were homozygous *sua4* and green seeds were heterozygous or homozygous *SUA4*. The phenotype of recombinants in the *SUA* region was confirmed by the ABA sensitivity of their progeny. By using a final mapping population of 3700 F3 plants (from a segregating F3 line equivalent to F2 plants), the position of the *sua4* mutation could be narrowed down to 64 Kb between 20.056 and 20.120 Mb on chromosome 3 (Figure 3.2.12). This region contains 17 loci, based on the Col genomic sequence (www.arabidopsis.org).

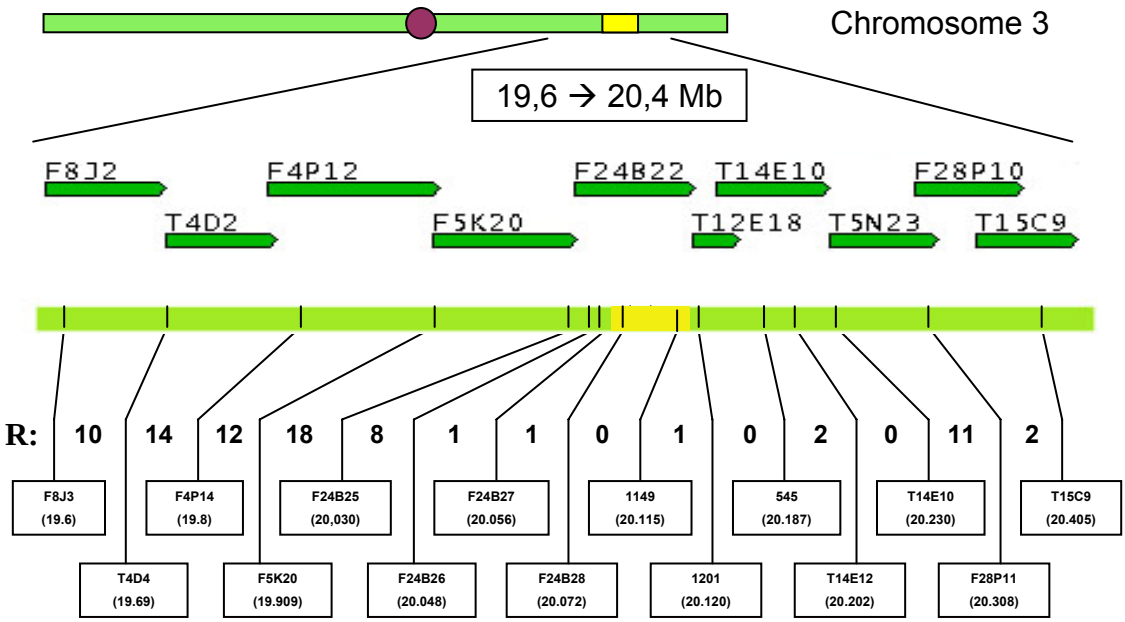


Figure 3.2.12: Fine mapping of the *sua4* mutation on chromosome 3 between 19.6 and 20.4 Mb. The *sua4* mutation was mapped in a 64 kb region between 20.056 and 20.120 Mb (shown in yellow), the BACs annotated at www.arabidopsis.org are depicted with dark green bars and a code. The molecular markers used for the fine mapping and their physical positions (Mb) are indicated in the boxes. The numbers in bold between the markers indicate the overall number of recombinants (**R**) identified within each interval among the 3700 F3 plants analyzed.

3.2.9 Genomic survey in the *sua4* region

In the 3700 plants that were used for the mapping, only 5 recombination events were detected in the region of 182 kb between the markers F24B26 and T14E10 (Figure 3.2.12). The average genome-wide recombination rate between Col and L*er* is estimated to be 260 kb/cM (Singer et al., 2006), which means that in a segregating population of 3700 individuals, within a region of 182 kb, about 52 recombinants are expected. The vicinity to centromeres, but also chromosomal rearrangements such as inversions, large insertions or

deletions can inhibit recombination during meiosis (Brenner et al., 1985). A region of approximately 150 Kb between the markers F24B27 (20.056 Mb) and T14E12 (20.202 Mb) was analyzed for major structural differences between the genomes of *sua4* (in L*er* background), L*er* and Col wild-type. Overlapping DNA fragments of approximately 5 Kb each, that covered the distance between F24B27 and T14E12, were amplified by PCR. The products were separated on 1% agarose gels. All the segments could be amplified, indicating that there were no major chromosomal differences between *sua4*, L*er* and Col. Therefore the gamma ray treatment did not cause major sequence alterations in the *sua4* genome. In one case, the analysis of an amplification product that covered the intergenic region between At3G54270 and At3G54280 revealed a deletion of 800 bp in L*er*, compared to Col. However, this difference would not be sufficient to explain the reduced recombination rate.

With the sequencing procedures used to identify the *sua4* mutation (see next paragraph), more than 37 Kb of genome in the *sua4* region, including many introns and intergenic regions, were sequenced. This survey identified a very high number of single nucleotide polymorphisms between L*er* and Col genomes. For example, in the intergenic region between At3G54280 and At3G54290 14.8% of the nucleotides (36/243) were polymorphic. Recent genome wide studies in *Arabidopsis thaliana* estimated that on average seven to eight nucleotides per Kb are polymorphic (Schmid et al., 2005). The high degree of sequence diversity between L*er* and Col in the genomic region of *sua4* could be the cause of recombination suppression.

3.2.10 Identification of the *sua4* mutation

Fine mapping of *sua4* narrowed down its location (paragraph 3.2.8) to a 64 kb region that contains 17 genes (Table 3.2.1). The predicted function of these genes, as described in the Arabidopsis Information Resource (www.arabidopsis.org), reveals that several of them could have a role in the development of seeds and during maturation, which is the stage when ABI3 exerts its function. For example the AtG354150 gene codes for a putative embryo abundant protein and was therefore a candidate gene. It was sequenced to investigate if it contained a mutation in the *sua4* mutant. Other candidate genes were selected for sequencing because of their expression patterns. The online bioinformatics tool

genevestigator (Zimmermann et al., 2004, 2005; www.genevestigator.ethz.ch) was used to identify the genes that are expressed in developing seeds based on microarray experiments. The gene At3G54230 was chosen because of its putative role in RNA metabolism, and because it could be involved in the post-transcriptional regulation of seed development related genes.

Table 3.2.1: Annotated genes in the region from 20.056 to 20.121 Mb on chromosome 3, based on the Col sequence available at the Arabidopsis Information Resource (www.arabidopsis.org). The genes that have been sequenced are highlighted in red.

Gene name	Position on chromosome 3 (bp)	Description
AT3G54140	20056641-20059550	Proton-dependent oligopeptide transport (POT) family protein.
AT3G54150	20061622-20063793	Embryo-abundant protein-related.
AT3G54160	20064446-20066005	Syntaxin-related family protein.
AT3G54170	20067683-20070500	Encodes protein that binds FKBP12. This interaction is disrupted by FK506 but not by cyclosporin A.
AT3G54180	20070774-20072416	Arabidopsis homolog of yeast cdc2, a protein kinase that plays a central role in control of the mitotic cell cycle.
AT3G54190	20072506-20075132	Similar to unknown protein.
AT3G54200	20076643-20077538	Similar to unknown protein.
AT3G54210	20078525-20079413	Ribosomal protein L17 family protein; identical to 50S ribosomal protein L17, chloroplast precursor (CL17) (RPL17).
AT3G54220	20081136-20083758	Encodes a member of a novel family having similarity to DNA binding proteins containing basic-leucine zipper regions. Regulates the radial organization of the root.
AT3G54230	20084082-20091242	Nucleic acid binding; similar to RNA recognition motif (RRM)-containing protein.
AT3G54240	20091904-20093123	Hydrolase, alpha/beta fold family protein; similar to hydrolase, alpha/beta fold family protein.
AT3G54250	20093286-20095710	Mevalonate diphosphate decarboxylase, putative; similar to MVD1.
AT3G54260	20095988-20097741	Similar to unknown protein.
AT3G54270	20098060-20100824	Sucrose-phosphatase 3 (SPP3); similar to sucrose-phosphatase 1 (SPP1).
AT3G54280	20103339-20115133	ATP binding / DNA binding / helicase; similar to homeotic gene regulator.
AT3G54290	20115377-20117022	Similar to hypothetical protein.
AT3G54300	20119187-20121661	Member of Synaptobrevin -like protein family.

The candidate genes were sequenced, using the *sua4* genomic DNA as template. After sequencing eight genes and more than 37 Kb, a deletion of 47 bp within an exon in the 3´ part of the gene At3G54230 was detected (Figure 3.2.13). This mutation causes a frame shift and a premature stop codon. Therefore, AtG54230 was identified as the *SUA* gene (formerly called *SUA4*) and the *sua4* allele was renamed *sua-1*. The confirmation of At3G54230 as *SUA* is described in the next chapter.

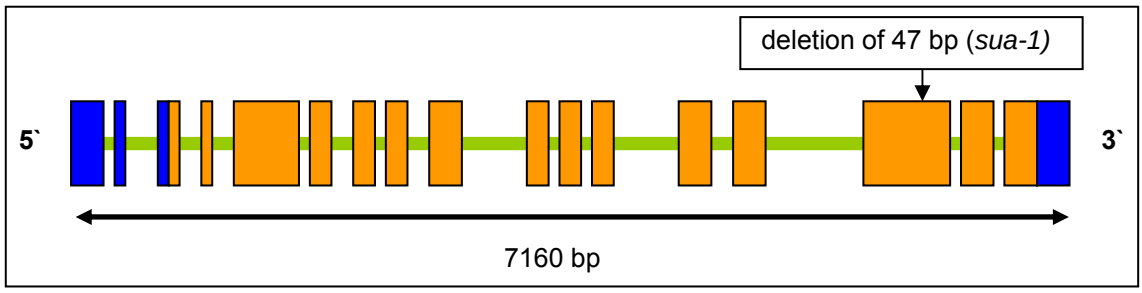


Figure: 3.2.13: Graphic representation of the *SUA* gene and the position of the *sua-1* mutation. The gene extends from 20084082 to 20091242 bp on chromosome 3. The untranslated regions are depicted in blue, the introns in green and the exons in orange.

3.3 Functional characterization of the *SUA* gene

3.3.1 Predicted SUA protein structure

The SUA protein was analyzed by using online bioinformatics tools, particularly the website of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). Two protein sequences corresponding to the *SUA* gene were found, annotated as the GenBank Identifiers (GI) 79444306 and 6822069. The latter protein corresponds to a 3315 bp long mRNA transcript which includes parts of the first and the twelfth introns. This suggests that *SUA* is alternatively spliced. The two annotated isoforms code for large proteins of 1007 and 1105 amino acids, characterized by similar domain architecture, consisting of two RNA recognition motifs (RRM) surrounding a Zinc finger domain (ZnF), and a glycine rich domain (G patch) close to the carboxy end (Figure 3.3.1). The conserved domain architecture of SUA, as identified by the CD software (Marchler-Bauer and Bryant, 2004; http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), is characteristic for proteins involved in RNA metabolism. The RRM domain is the most widespread eukaryotic RNA binding module and mediates RNA recognition in hundreds of proteins from the RNA processing machinery (Burd and Dreyfuss, 1994). Proteins involved in post-transcriptional regulation have a modular structure, being composed of RNA binding domains and domains that perform additional functions, for example in mediating protein-protein interactions. RRM functions primarily in targeting specific RNAs, but for the specificity of recognition auxiliary domains are required. RNA binding proteins provide specificity of interaction with components of the RNA processing apparatus, allowing functional proteins complexes to assemble on specific mRNAs (Varani and Nagai 1998). The G patch is a conserved domain of about 40 amino acids that has been found in a number of putative RNA binding proteins including the human tumor suppressor RBM5 (Drabkin et al., 1999), many RNA processing proteins such as the human 45-kDa splicing factor (Neubauer et al., 1998) and the type-D retroviral polyproteins (Aravind and Koonin, 1999). Another feature of SUA is the zinc finger domain between the two RRMs. This kind is a small protein motif with zinc ion chelating cysteines that stabilizes a finger-like-fold. It generally mediates binding to different substrates, including RNA (Laity et al., 2001).

		Protein Sequence		RRM-ZnF-RRM		G patch	
	GI	Lenght	Similarity	Position	Identity	Position	Identity
Arabidopsis thaliana	79444306	1007	-	271-535	-	917-983	-
Vitis vinifera	157341988	989	70%	262-506	74%	873-985	71%
Oryza sativa	49387752	928	65%	166-411	69%	779-897	61%
Physcomitrella patens	168014858	888	65%	112-576	51%	726-887	50%
Homo sapiens	13124794	815	45%	73-353	26%	741-814	41%
Xenopus tropicalis	134023691	838	41%	39-563	24%	719-837	34%
Aspergillus fumigatus	159127876	722	40%	118-385	40%	599-687	40%

Figure 3.3.1: Schematic representation of the SUA protein predicted structure and the conserved domains: in red the two RNA binding motifs, in green the Zinc finger domain and in blue the Glycine rich domain. Homologous eukaryotic proteins with similar architecture, as annotated in the GenBank, are shown below the cartoon. Each protein sequence is described by length (in amino acids) and the degree of similarity to the SUA sequence. The cluster of motifs including the two RRM and the ZnF domains, and the G patch domain are described by their relative position within the corresponding protein and the percentage of conserved amino acids compared to SUA.

3.3.2 Identification, cloning and sequencing of *SUA* splice variants

The *SUA* transcript in developing siliques of L*er* and *sua-1* plants, harvested 14 days after pollination, was analysed by Reverse Transcription (RT) PCR. The cDNA species corresponding to the *SUA* transcripts were amplified with primers annealing to the 5′and 3′ terminal coding regions of the *SUA* gene. The mutation in *sua-1* does not prevent transcription and the mutant gene encodes for a truncated SUA protein which misses the amino-terminus, including the G Patch domain. Separation on agarose gel of the amplified cDNAs revealed the existence of at least two splice variants (Figure 3.3.2). The most abundant RT-PCR product has a size of 3021 bp, coding for the 1007 amino acids SUA. The splice variant corresponding to the 1105 amino acids long protein annotated in the GenBank sequence database (GI: 6822069) could not be found. Separation on gel of the RT-PCR products of the *SUA* cDNA highlighted the bands of at least two splice variants with a larger size than the 3021 bp species, which were more abundant in L*er* than in *sua-1* (Figure 3.3.2 A). Zooming in the 3′ region of *SUA* with RT-PCR, three fragments were amplified (Figure 3.3.2 B). The amount of the wild-type 3021 bp and the *sua-1* partially deleted 2974 bp transcript species did not seem to vary between the two samples (Figure 3.3.2 A and B, corresponding to the strongest bands).

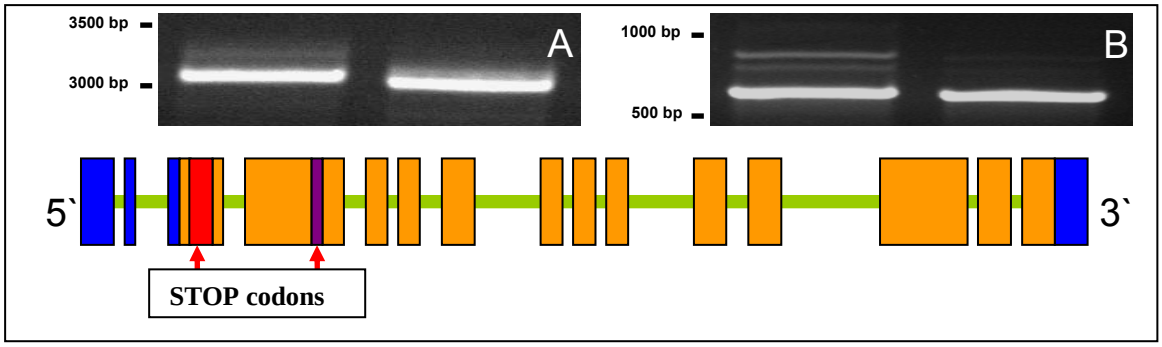


Figure 3.3.2: Gel pictures of the RT-PCR products of *SUA* cDNA. (A): amplified products of the *SUA* complete cDNA from L*er* template (left) and from *sua-1* (right). (B): amplified fragments corresponding to the 5`region of *SUA* cDNA from L*er* (left) and *sua-1* (right). The bands of higher intensity in both pictures correspond to the 3021 bp *SUA* transcript (for L*er*) or 2974 bp (for *sua-1*) Below, the schematic representation of the *SUA* gene: in orange the exons composing the 3021 bp most abundant transcript. The introns that are maintained in two distinct splice variants that have been cloned are marked in red and violet.

The pre-mRNAs of many spliceosomal proteins are consistently alternatively spliced (Isshiki et al., 2006) in a developmental and tissue-specific manner. Stress can also influence splicing, but the splice variants do not necessarily have biological functions. Two splicing forms of *SUA* were extracted from the agarose gel and cloned into pCR® -Blunt II-TOPO® amplification vector. Sequencing of these fragments showed that they included respectively the third and the fifth intron. Both variants code for a truncated protein: the introns, incorporated in the mature mRNAs, introduce stop codons in the 5` region of the transcripts (Figure 3.3.2). The reduced abundance of alternatively spliced *SUA* variants in the *sua-1* mutant (Figure 3.3.2) might indicate that the SUA protein is necessary for its own alternative splicing. Another possibility is that the mutant *sua-1* mRNA, due to the deletion of 47 nucleotides in the 3`region, has a reduced recognition by the splicing machinery.

3.3.3 The influence of *SUA* on the expression of transcription factors involved in seed development

The results in paragraphs 3.3.1 and 3.3.2 suggest that the *SUA* gene is involved in alternative splicing. The suppression effects of the *sua-1* mutation on the *abi3-5* mutant phenotype described in chapter 3.2, might be the consequence of an alteration in the post-transcriptional regulation of seed specific genes. Several transcription factors act simultaneously with ABI3 during maturation (Parcy et al., 1997; Mönke et al., 2004; Lara et al., 2003; Vicente-Carbajosa and Carbonero, 2005; Kroj et al., 2003). *ABSCISIC ACID INSENSITIVE 5* (*ABI5*) is a bZIP transcription factor (Finkelstein and Lynch, 2000), which binds specific elements in the promoters of *LATE EMBRYO ABUNDANT Em1* and *Em6* genes (Carles et al., 2002). ABI5 interacts with ABI3 (Nakamura et al., 2001) and contributes to the ABI3 dependent activation of maturation specific genes. Another bZIP transcription factor: *ENHANCED EM LEVEL (EEL)* can bind the same promoter motifs as ABI5, but it acts antagonistically, suppressing the transcription of *Em1* and *Em6* (Bensmihen et al., 2002). *EEL* is expressed in three isoforms and therefore a good candidate for having possible modifications in the alternative splicing processes in the *sua-1* mutant. The *HEAT SHOCK TRANSCRIPTION FACTOR A9* (*HSFA9*) acts downstream of ABI3 and specifically activates the transcription of heat shock proteins necessary for the establishment of desiccation tolerance in seeds (Wehmeyer and Vierling, 2000). Its expression is controlled by the ABI3 protein that specifically binds the RY motifs (CATGCATG), *cis* acting elements present in the *HSFA9* promoter (Kotak et al., 2007). The expression of the *ABI5*, *EEL* and *HSFA9* genes was analyzed by semi-quantitative RT-PCR on cDNA samples of L*er*, *abi3-5*, the *sua-1 abi3-5* double mutant and *sua-1* single mutant, obtained from siliques harvested during seed maturation (14 days after pollination). The abundance of *ABI5* and *EEL* transcripts, and of the splice variants of *EEL1* was very similar in all cDNA samples (Figure 3.3.3). The *HSFA9* transcript was detected in the L*er* and *sua-1* samples, but it was absent in the *abi3-5* cDNA pool, confirming the findings of Kotak et al. (2007). A weak but significant expression of *HSFA9* was detected in the *sua-1 abi3-5* (Figure 3.3.3), indicating that in the double mutant the molecular functions of ABI3 were partially restored.

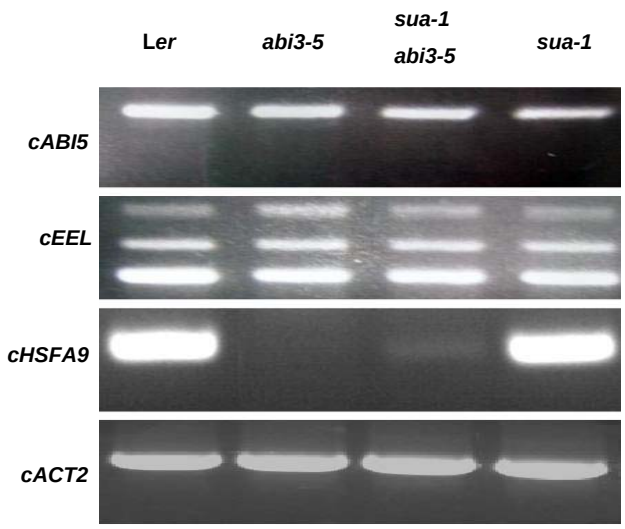


Figure 3.3.3: RT-PCR expression analysis of the seed specific transcription factors *ABI5*, *EEL* and *HSFA9*. The template cDNAs were obtained from developing siliques harvested 14 days after pollination. The primer combinations amplified a 649 bp fragment of the *ABI5* transcript (28 cycles), 670, 578 and 518 bp fragments of *EEL* splice variants (28 cycles) and a 202 bp fragment of *HSFA9* transcript (32 cycles). A 600 bp fragment of the *ACTIN2* transcript was amplified as control (28 cycles).

3.3.4 Localization studies of *SUA* expression

In order to better understand the *SUA* gene function its expression pattern was analyzed. The microarray data available at the website: https://www.genevestigator.ethz.ch/ (Zimmermann et al., 2004 and 2005) shows ubiquitous expression of *SUA*. The relative abundance of *SUA* transcripts is constant throughout the Arabidopsis life cycle (Figure 3.3.4). To confirm this, a binary vector carrying the *SUA* promoter (-2711 bp), controlling the *β-GLUCURONIDASE* (*GUS*) gene was generated. Agrobacterium-mediated Arabidopsis plant transformation yielded several transformants and four independent T2 homozygous lines carrying a single copy insertion were analysed. GUS staining could be detected in all tissues after treatment with the GUS reagent, confirming that the *SUA* promoter is active in all tissues (Figure 3.3.4).

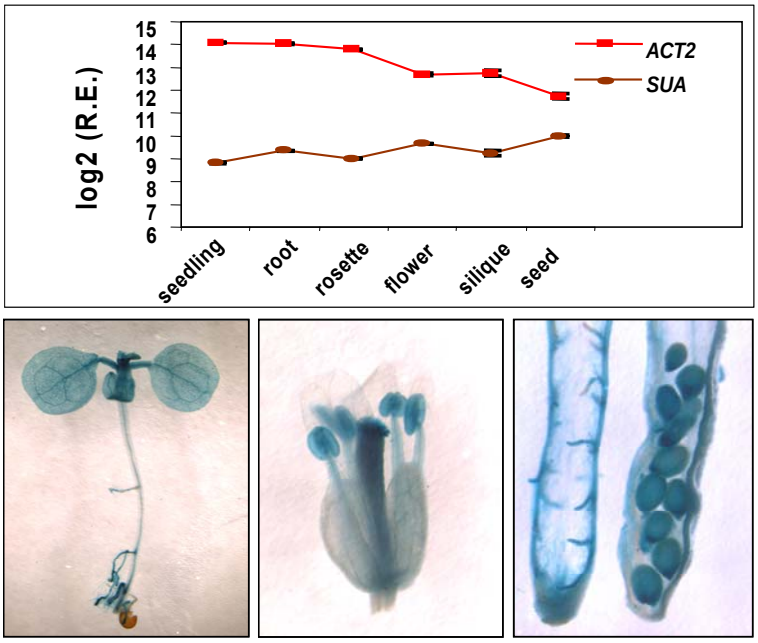


Figure 3.3.4: The graph represents the logarithmic relative expression (R.E.) in different tissues of the *SUA* gene (brown line) and the *ACTIN2* control gene (*ACT2*, red line). Microarray data were obtained from genevestigator database.
The photos show GUS staining from transgenic Arabidopsis tissues expressing the *GUS* gene under the control of the *SUA* promoter (-2711 bp).

3.3.5 Cellular localization of SUA_GFP

Based on its predicted functions (paragraph 3.3.1), the SUA protein is expected to be localized in the nucleoplasm. A binary vector with the *SUA* cDNA under the control of a 35S promoter and fused at the 3′ end with the *GREEN FLUORESCENT PROTEIN* (*GFP*) gene was used for the transient overexpression of *SUA_GFP* in *Nicotiana benthamiana* leaves. Two days after inoculation, cells displaying fluorescent nuclei were spotted. The fluorescence pattern inside the nuclei showed speckles of fluorescence, indicating the presence of aggregates of the SUA_GFP chimeric protein (Figure 3.3.5). Propidium Iodide staining of the same tissue was used to highlight the nucleic acids and confirm that the GFP signal was located in the nucleus.

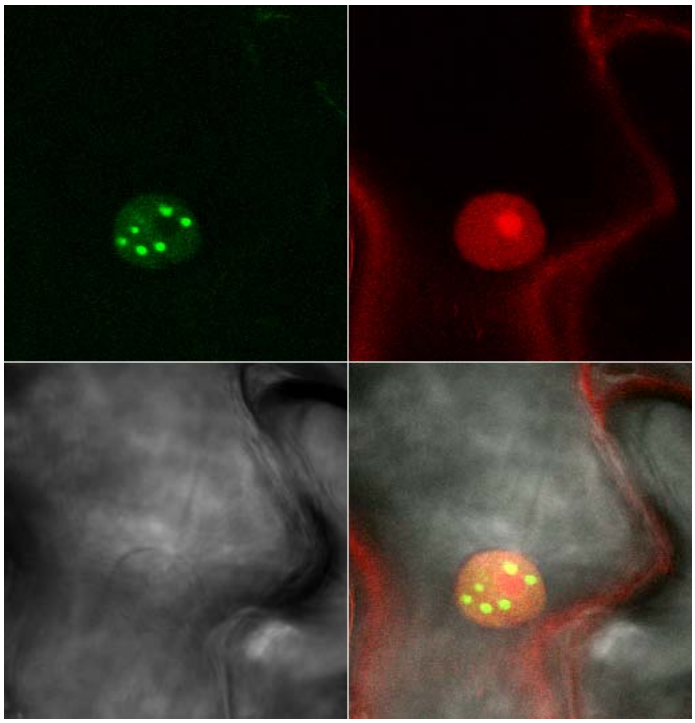


Figure 3.3.5: Confocal laser microscope picture of a *Nicotiana benthamiana* leaf cell overexpressing SUA_GFP fusion protein. Top left: GFP emission (peak wavelength 512 nm). Top right: staining with propidium iodide of nucleus, nucleolus and membranes (peak emission 615 nm). Bottom left: bright field image. Bottom right: overlap.

A construct for Agrobacterium mediated plant transformation carrying the *SUA_GFP* fusion gene under the control of the native *SUA* promoter (-2711 bp) was used to transform Arabidopsis. Many transgenic lines that exhibited nuclear fluorescence were obtained. In some cells the GFP signal had patterns similar to those already observed in the transient *SUA_GFP* overexpression assays (Figure 3.3.5), although the fluorescent punctuated structures appeared less marked and more numerous. In some nuclei the fluorescence signal was diffused and rather weak and only a few nuclei of the analyzed tissues were fluorescent (Figure 3.3.6). It was previously shown (paragraph 3.3.4) that the *SUA* promoter is constitutively active in every tissue, but cell specific regulation of expression cannot be excluded. The microscopic observations indicate that *SUA* might be tightly controlled at the post-transcriptional level, a hypothesis in agreement with the finding of nonsense splice variants (see Paragraph 3.3.2). Similar fluorescence patterns were observed in animal cells expressing GFP tagged YT521-B, a nuclear protein involved in splicing site recognition that forms aggregates specifically during the G1 and M phases of the cell cycle (Nayler et al., 2000). The analyzed cells were from developing seed radicles, which were most likely not synchronized. The GFP signal patterns might mark cells in a specific phase of their cycle (Figure 3.3.6).

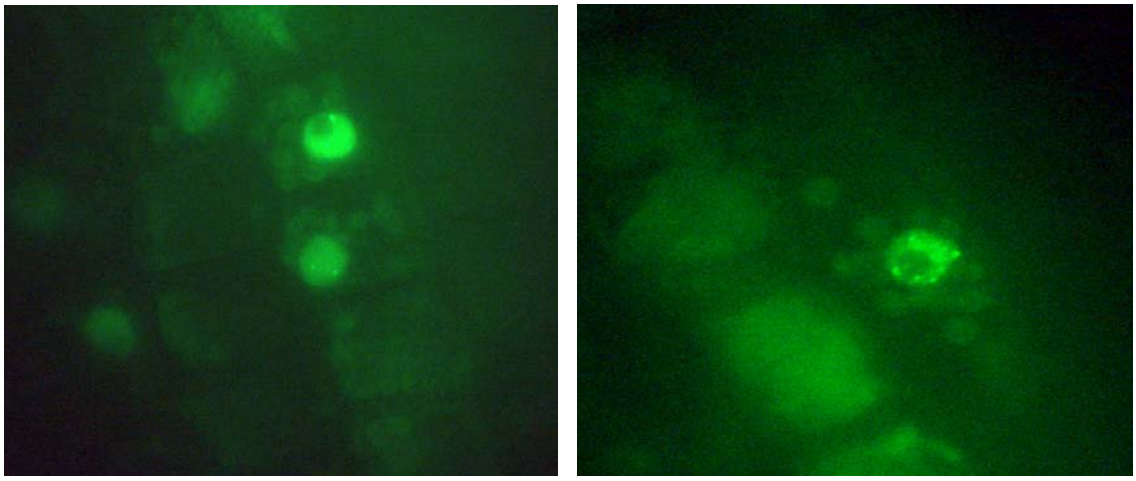


Figure 3.3.6: Fluorescence microscope images of developing embryo root tissues from transgenic plants expressing SUA_GFP under the control of the native *SUA* promoter. The GFP excitation was provided with UV light (395 nm) and emission light (510-560 nm) was filtered through a dichroic mirror.

3.3.6 Heterologous expression of SUA protein

In order to understand the SUA functions at the molecular level, in vitro activity experiment were planned. One of the aims was to investigate the nucleic acid binding capabilities with particular regard to the putative interaction between the SUA protein and seed specific transcripts, like *ABI3* or *HSFA9*. Another hypothesis to be tested is that the SUA protein might act antagonistically to transcription factors at the DNA level, binding *cis* acting elements of seed specific promoters as a suppressor of expression. There is experimental evidence that certain RRMs can bind DNA and RRMs have been found in the structures of transcription factors (Basu et al., 1997; Hamimes et al., 2006). Furthermore SUA is characterized by a Zinc finger motif, which could also bind DNA (Laity et al., 2001). Therefore, in vitro DNA binding assays were planned. The SUA protein tagged with 6XHis at the amino terminus was expressed into *E. coli* with an inducible overexpression vector (paragraph 2.2.26) and purified (Figure 3.3.7). SUA protein was obtained, but other experiments were given priority and therefore the follow up work is not part of this thesis.

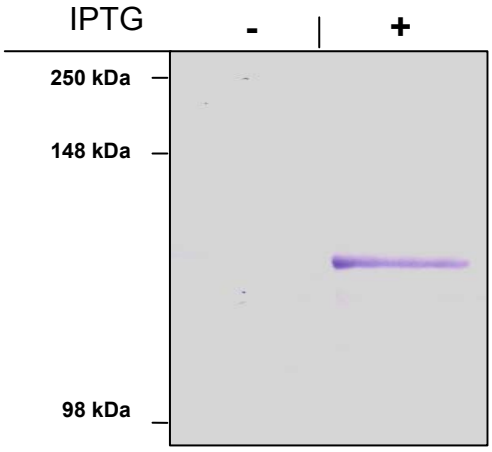


Figure 3.3.7: Detection of His-tagged SUA protein of 118.8 kDa, purified from E. coli cell culture. Proteins were extracted from overnight grown cultures (at 20 °C). Transgenic E. coli was grown without induction (-) or in the presence of 0.15 μM IPTG (+). The tagged SUA protein was purified and detected on gel after hybridization with a poly His specific Ni-NTA conjugated with the Alkaline Phosphatase enzyme.

3.3.7 Complementation of the *sua-1* mutant

To confirm the identity of At3G54230 as *SUA*, complementation experiments were performed by transformation of the *sua-1 abi3-5* double mutant. The genomic sequence of At3G54230 and its promoter was subcloned from a bacterial artificial chromosome clone into a binary vector (paragraph 2.2.20). The construct was used for the Agrobacterium-mediated transformation of 120 *sua-1 abi3-5* plants. The transformation experiment yielded only one transgenic plant that segregated in a 3:1 ratio for the green seed phenotype typical of the *abi3-5* mutant. This result indicates that introducing the functional allele of the *SUA* gene into the double mutant genetic background complements the suppression effects of *sua-1*.

The binary vector used for the complementation experiment contains the complete genomic region of *SUA* plus the intergenic regions surrounding it with a total size of about 14 kb, the low transformation efficiency observed might be due to the type of binary vector used. Furthermore the T1 seeds hemizygous for the transgene had a poor longevity typical for *abi3-5* mutants and many might have died prior to sowing.

Transformation of the *sua-1 abi3-5* double mutant with the overexpression vector carrying the *SUA* cDNA sequence under the control of the 35S promoter did not yield any transgenic plant. The same result was obtained with the overexpression of the *SUA_GFP* chimeric gene. Four transformation experiments with *sua-1 abi3-5* double mutant and wild-type plants were unsuccessful, suggesting that the ectopic expression of *SUA* might lead to lethality. This hypothesis is supported by recent studies on the human homologue

of *SUA*, *RBM5* (paragraph 3.3.1) which has been described as a tumor suppressor gene that controls apoptosis (Oh et al., 2006).

More than a hundred *sua-1 abi3-5* T1 transgenic lines showing the typical *abi3-5* seed phenotypes were obtained after transforming the double mutant with the construct carrying the *SUA_GFP* gene under the control of the native *SUA* promoter. The vector used for this purpose (pBAT B) proved highly efficient for plant transformation but mainly produces lines with multiple T-DNA insertions.

Genomic DNA of the At3G54230 gene, as well as its cDNA under the control of the native promoter, complements the *sua-1* mutant phenotype in the *abi3-5* background and confirms that At3G54230 is the *SUA* gene.

3.3.8 Phenotypes of *sua-1* in Ler wild-type background

The *sua-1* mutant allele was separated from *abi3-5* using a marker assisted selection. The *sua-1* phenotype in wild-type background was analyzed to understand if the *SUA* gene has a general role in seed maturation. The *sua-1* plants developed normally and yielded seeds that were slightly lighter than L*er* seeds (*sua-1* 14.27±1.05 μg/seed, L*er* 15.48 ± 0.98 μg/seed, as an average of five biological replicates). Furthermore, they were characterized by increased dormancy, and increased sensitivity to ABA (Figure 3.3.8). These two aspects are in contrast with *abi3-5* phenotypes and are therefore consistent with the expected effects of an *abi3* suppressor mutant. The *sua-1* mutant seeds proved less resistant than L*er* to controlled deterioration (data not shown), and reduced longevity was also observed in germination tests conducted after long term storage (Figure 3.3.8). This result was unexpected since the characteristic phenotype of the *sua-1 abi3-5* double mutant is the improved longevity compared to *abi3-5*.

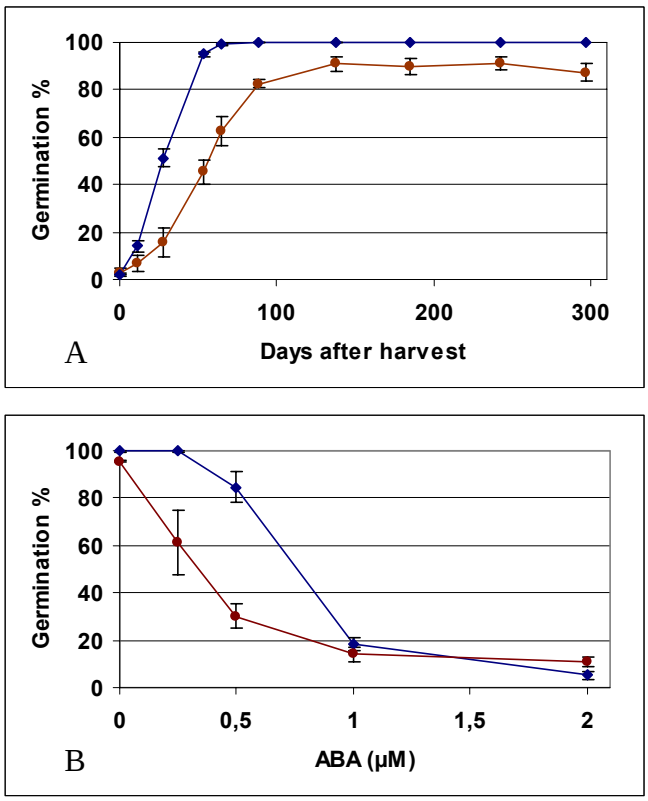


Figure 3.3.8: (A): Dormancy phenotype of the *sua-1* single mutant. L*er* seeds (blue line) released dormancy during the first 60 days of storage, while *sua-1* seeds (brown line) were slower and started to die after 100 days of storage. The data points are the average of 10 biological replicates, standard error bars are indicated.
(B): Abscisic acid (ABA) sensitivity at germination for L*er* (blue line) and *sua-1* (brown line). Seeds were sown on different ABA solutions and stratified prior to germination. Data points are the average of five biological replicates, standard error bars are indicated.

3.3.9 The influence of *sua-1* in other seed developmental mutant backgrounds

The 47 bp deletion in the *SUA* gene, defining the *sua-1* mutant allele, was used to generate a PCR marker for genotyping the *sua-1* mutation (Table 3.3.1 in Appendix). This marker was used to select the double mutant in the F2 of a cross between the *sua-1* mutant and other seed development mutants. *LEAFY EMBRYO COTYLEDON1* (*LEC1*), *LEC2* and *FUSCA3* (*FUS3*) are transcription factors that act in concert with *ABI3* during seed development and maturation (To et al., 2006). The *lec1-3*, *fus3-1* and *lec2-1* alleles are recessive loss of function mutations that cause morphological alterations in the embryo, accumulation of anthocyanins, precocious germination, reduced storage reserves and in general very short longevity (Holdsworth et al., 1999; Finkelstein et al., 2002). Homozygous double mutants combining *sua-1* with *lec1-3*, *fus3-1* and *lec2-1* alleles were obtained to assess if *sua-1* could suppress any of the phenotypes of these mutants. The progenies of each double mutant were tested for germination a few weeks after harvest and proved less resistant to aging than the single mutants seeds (data not shown). The accumulation of anthocyanins in *sua-1 lec1-3* and *sua-1 fus3-1* seeds was increased, while *sua-1 lec1-3* and *sua-1 lec2-1* double mutant seeds were characterized by vivipary. At maturity the siliques of these lines were often filled with roots, an enhancement of the

phenotype of *lec1-3* similarly to what observed in *abi3 lec1* double homozygotes (Meinke et al., 1994). These results suggested that the *sua-1* mutation caused a reduction of seed quality in the other mutant backgrounds that we tested. This phenotype of *sua-1* in seed developmental mutants is opposite to the suppression effects observed in the *abi3 sua1* double mutant.

3.3.10 Study of new *SUA* mutant alleles

Two T-DNA insertion lines were obtained From the Nottingham Arabidopsis Stock Centre. The homozygous mutant lines SALK_054379 and GABI-Kat 815C12 were selected by genotyping the provided T2 populations with specific PCR markers annealing to the T-DNA border (reverse primer) and the *SUA* gene (forward primer). The new *sua* alleles were named *sua-2* and *sua-3* alleles respectively and are characterized by a T-DNA insertion in the fourth and the ninth introns respectively (Figure 3.3.9). The cDNA obtained from developing siliques of the homozygous *sua-2* line was tested by RT-PCR and did not contain any signal for *SUA*. Therefore, this allele was considered to be a loss of function mutation. To study these new alleles and to verify that the loss *SUA* gene function was responsible for the suppression of *abi3* phenotypes, *sua-2* and *sua-3* were crossed to *abi3-6*, a phenocopy of the *abi3-5* mutant allele (Nambara et al., 1994, figure 3.3.10). The *abi3-6* mutant was also chosen because it is in the Columbia background, like the T-DNA insertion lines, so the progenies of the crosses would segregate for the mutant alleles in a homogeneous genetic background. The F2 plants which were homozygous for the *sua* T-DNA alleles and *abi3-6* yielded seeds that did not display any *abi3* suppression phenotypes; retaining chlorophyll at maturity, quickly losing viability and being strongly insensitive to ABA. Furthermore, *sua-2* and *sua-3* single mutant seeds did not show the differences in dormancy and ABA sensitivity when compared to wild-type Col seeds, unlike the comparison of *sua-1* with L*er* (paragraph 3.3.9). This discrepancy could be explained by the different genetic backgrounds. SUA might be part of a large network of RNA binding proteins with redundant functions for the RNA processing. In this scenario the Columbia genetic background might contain modifiers of the *sua* mutations effects. It is also possible that *sua-1* is translated into a truncated protein with the RRMs and the Zn finger, but missing the G patch domain at the carboxy terminous, which could compete for

substrates with the mRNA processing machinery, hindering its functions as a dominant negative allele. However, a significant result was obtained when *sua-2* and *sua-3* were combined with *abi3-5* in L*er* background. These double mutants showed suppression of the *abi3-5* phenotypes, similar like in the *sua-1 abi3-5* double mutant. This showed that all three available *sua* mutant alleles act as suppressors of *abi3-5*.

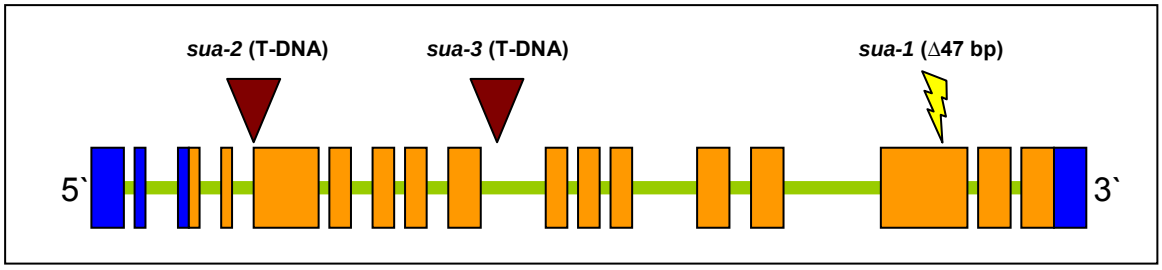


Figure 3.3.9: Graphic representation of the different mutations in the *SUA* gene. The UTRs are shown in blue, the exons in orange and the introns in green. The SALK insertion line 054379 (*sua-2*) is characterized by a T-DNA insertion at the splicing site before the third exon. The GABI-Kat line 815C12 (*sua-3*) has the T-DNA insertion in the ninth intron. The *sua-1* mutation is induced with gamma rays and consists of a 47 bp deletion in the thirteenth exon.

3.3.11 The *sua* mutants are allele specific suppressors of *abi3-5*

The *sua-1* mutant was crossed with *abi3-6* and the double mutant was selected. Similar to the double mutants of *sua-2* and *sua-3* with *abi3-6*, the *sua-1 abi3-6* double mutant did not show any suppression of the *abi3-6* phenotype. Suppression phenotypes for all *sua* mutants were only observed in *abi3-5*, showing that they are allele specific. This issue was further investigated by the study of additional *abi3* mutant alleles (Figure 3.3.10). The strong ABA insensitive *abi3-4* allele, as well as the leaky *abi3-1* and *abi3-7* alleles were combined with *sua-1*. All these combinations did not show any suppression phenotypes. The *sua-1 abi3-4* double mutant produced seeds with the typical strong *abi3* mutant phenotype. Double mutant seeds of *sua-1 abi3-1* and *sua-1 abi3-7* were tested for their sensitivity at germination to the ABA hormone. They did not show any significant difference compared to the *abi3-1* and *abi3-7* single mutants (data not shown).

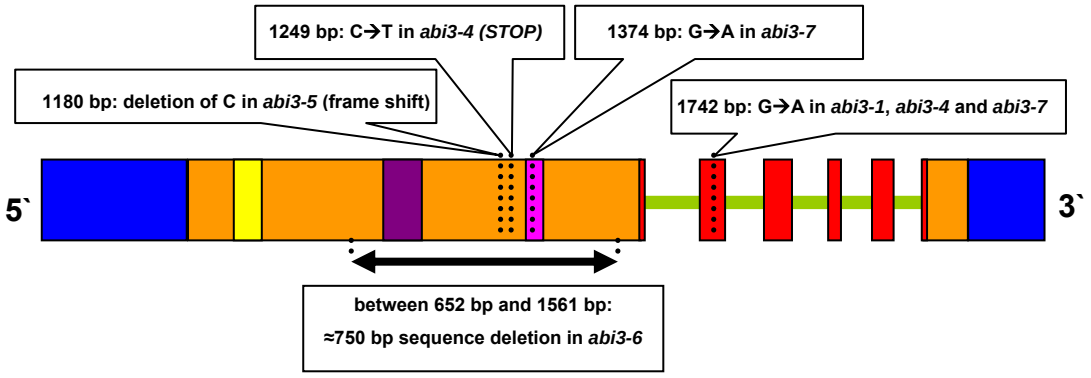


Figure 3.3.10: Representation of the *ABI3* gene. The untranslated regions are shown in blue, the introns in green, the exons in orange, the A1 activation domain (216-372 bp) in yellow, the B1 domain (729-930 bp) in violet, the B2 domain (1335-1419 bp) in pink and the B3 domain (1670-2027 bp) in red. The location and nature of the *abi3-1*, *abi3-4*, *abi3-5*, *abi3-6* and *abi3-7* mutations are indicated (the nucleotide positions refer to the coding sequence).

3.3.12 Detection of ABI3 protein

Former studies showed that the *abi3-4* and *abi3-6* mutants produce an *ABI3* transcript, and that in *abi3-4* a truncated ABI3 protein is highly expressed (Parcy et al., 1997; Figure 3.3.11). *ABI3* expression could also be detected in *abi3-5*. Therefore, the possibility that the *abi3-5* transcript in the *sua-1* genetic background could be modified into a functional *ABI3* mRNA was investigated. The RT-PCR analysis of L*er*, *sua-1*, *abi3-5* and the double mutant *sua-1 abi3-5* did not highlight differences in the abundance of the *ABI3* and *abi3* transcripts (data not shown). Sequencing of the complete *abi3* cDNA amplified from the double mutant cDNA pool showed that the transcript was consistent with the *abi3-5* mutation, which is a deletion of the cytosine in the first exon of the *ABI3* gene (see Figure 3.3.10). The influence of *sua-1* on the abundance of ABI3-5 mutant or ABI3 proteins was analyzed by western immunoblot analysis. Proteins were extracted from dry seeds of L*er*, *sua-1*, *abi3-5*, *sua-1 abi3-5*, *abi3-4* and *sua-1 abi3-4*, and for detection an antibody targeted to the amino end of the ABI3 protein was used, kindly provided by Kazumi Nakabayashi (Nakabayashi and Nambara, RIKEN plant science center, Yokohama, Japan), (Figure 3.3.11).

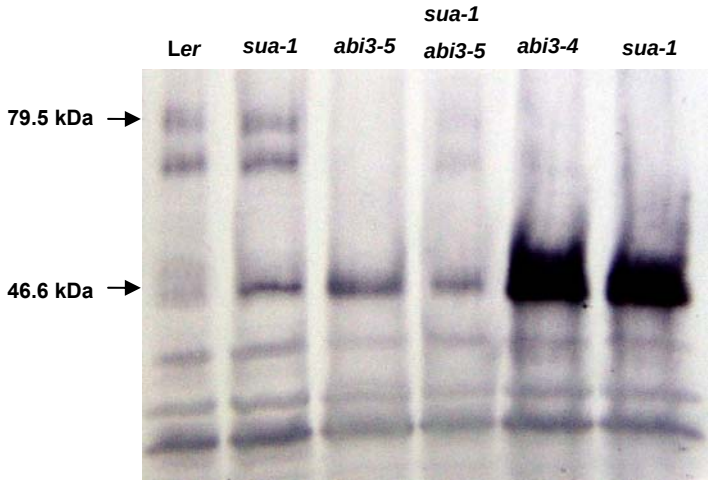


Figure 3.3.11: Immunoblot analysis of ABI3 in 120μg seed protein extracts. Proteins were separated on a tris-glycine 4-12% acrylamide gradient gel. The ABI3 protein (79.5 kDa) is identified as a double band in L*er*, *sua-1* and the *sua-1 abi3-5*. Truncated ABI3 forms are identified in all protein samples except L*er*. Unspecific bands can be observed in the lower part of the gel.

The western analysis showed two bands for the ABI3 in L*er* and *sua-1* samples, which might be due to partial degradation or modification of the ABI3 protein. A truncated ABI3 protein of about 46.6 kDa (corresponding to 428 aa polypeptide) can be observed in the *abi3-5* mutant. A similar size truncated ABI3 protein was found in *abi3-4* (416 aa, 45.1 kDa) and *sua-1 abi3-4*. In the *sua-1 abi3-5* double mutant two weak bands corresponding to ABI3 could be detected (720 aa, 79.5 kDa), along with the lower band spotted in the *abi3-5* single mutant. The presence of a full lenght ABI3 protein in the double mutant was consistent with all the observed suppression effects described for *sua-1 abi3-5* in Chapter 3.2. Presence of full length ABI3 protein was an unexpected result because it necessitates the neutralization of the *abi3-5* allele mutation, which consists of a single nucleotide deletion causing a shift of the reading frame and an early stop codon.

In the *sua-1* seed protein extract, along with the bands corresponding to the ABI3 wild-type protein, a band corresponding to the size of the truncated ABI3 protein present in the *abi3-5* and *abi3-4* mutants was detected. The presence of an aberrant ABI3 protein can explain the seed phenotypes observed in the single *sua-1* mutant seeds.

3.3.13 The *sua* mutant contains a new *ABI3* splice variant

The full lenght ABI3 protein in the *sua-1 abi3-5* double mutant seeds must be translated from a functional *ABI3* transcript without the early stop codon that characterizes the *abi3-5* allele (Figure 3.3.10). Previous analyses (paragraph 3.3.12) did not show any alteration of the *abi3-5* transcript, and the ABI3-5 truncated protein was also detected in the double

mutant seeds. Therefore it is possible that a minor fraction of the *abi3-5* transcript is modified by the *sua* mutation, allowing the accumulation of a functional ABI3 protein. The *abi3-5* allele causes a frame shift and the formation of a premature stop signal after 34 erroneous codons. A more detailed RT-PCR analysis of the transcript region around the position of *abi3-5* mutation was conducted. Using the primer combination mabi351f-353r, which anneals at position 1120 and 1718 bp of the *abi3-5* "coding" sequence, and saturating RT-PCR conditions (35 cycles), two fragments of different size could be amplified from the *sua-1 abi3-5* cDNA template. Separation on agarose gel showed a faint band of smaller size and a more abundant larger band (Figure 3.3.12). The new fragment was purified and sequenced, which revealed that it corresponded to a novel *abi3* transcript species, where a region of 77 bp, located 57 bp downstream to the *abi3-5* deletion, and including the position of the *abi3-5* premature stop codon, was absent. The missing fragment has the sequence features of an intron, particularly at the two borders (Figure 3.3.12), while it lacks a polypirimidine stretch in 3` that generally characterizes introns (Reddy, 2007).

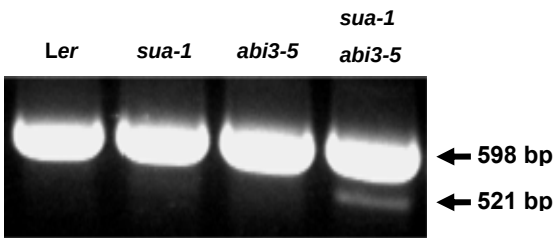
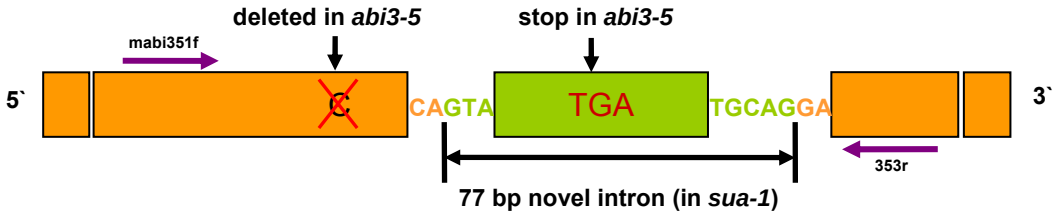


Figure 3.3.12: RT-PCR amplified fragments from the first exon of *ABI3* and *abi3-5*. Two bands are visible in the *sua-1 abi3-5* double mutant. Below: graphic representation of the *abi3-5* transcript region and the two primer annealing sites (mabi351f and 353r) used to identify the novel splice variant. The position of *abi3-5* mutation is indicated and the cryptic intron is shown in green.



The new transcript identified in the *sua-1 abi3-5* double mutant is therefore a novel splice variant of *ABI3*; the *sua-1* mutation is responsible for the accumulation of a new apparently functional *abi3* mRNA species. The removal of the cryptic intron of 77 bp, in combination with the single nucleotide deletion of *abi3-5*, results in a transcript that restores the reading frame of *abi3-5* after 21 erroneous and 26 deleted codons. The corresponding translated polypeptide is a new ABI3 protein (called ABI3-5SV) of 694 aa and 76.74 kDa that, in agreement to the phenotypes of the *sua-1 abi3-5* seeds, maintains most of the ABI3 molecular functions. The results described in this paragraph explain the allele specific suppression observed in *sua-1 abi3-5*. Only the combination of a single nucleotide deletion in the vicinity of the novel intron can maintain the reading frame in the newly identified splice variant. In a wild-type background, *sua-1* causes the splicing of a portion of the *ABI3* transcript. The novel splice variant has a frame shift after the intron splice site and an early stop codon after 16 erroneous amino acids. Consequently, a fraction of the *ABI3* transcript in the *sua-1* background codes for a truncated ABI3 protein. This is consistent with the immunoblot analysis of the protein extract from *sua-1* single mutant seeds, that contains the ABI3 wild-type protein and in addition a 429 aa long ABI3 polypeptide of 46.6 kDa (Figure 3.3.11).

4 Discussion

4.1 Factors influencing seed longevity

4.1.1 Environmental factors

Developing seeds are a sink of plant nutrients. The environment can influence the ability of the plant to supply these nutrients and to regulate their accumulation in the seeds, which determines seed quality traits, including longevity. Furthermore the maternal environment can also affect the hormone levels, which can lead to variation between seeds from the same plant in for instance abscisic acid (ABA) levels (Chono et al., 2006), the key hormone required for seed maturation (Nambara and Marion-Poll, 2003). Because every single seed is subjected to different interactions during its development, in a population of orthodox seeds the distribution of individual life spans shows a normal distribution.

Seeds from the Arabidopsis *abi3* and *lec1* mutants have a reduced response to ABA (Parcy et al., 1997), and an abolished maturation phase, which causes an extreme sensitivity to desiccation. During the ripening of orthodox seeds the moisture content is progressively reduced. In *lec1-3* and *abi3-5* desiccation sensitive seeds, this causes an immediate damage resulting in a rapid loss of viability. The drying speed depends on the conditions of temperature (T) and relative humidity (RH) surrounding the plant, and on the water availability of the soil. The viability of *abi3-5* and *lec1-3* mutant seeds immediately after harvest varied from 0 to 80% depending on the experiment. Major seasonal differences in longevity have been observed in the progenies of *abi3-5* and *lec1-3* mutant plants grown in the greenhouse. Seeds harvested in summer showed a lower viability immediately after harvest and after storage at room conditions, compared to seeds harvested in winter. The desiccation sensitive seeds from *lec1-3* and *abi3-5* plants were extremely sensitive to small environmental differences because seed batches obtained from plants with the same mutant genotype grown simultaneously could show a large variation in seed longevity.

4.1.2 Storage conditions

It has been previously determined that more than 80% of dry mature wild-type seeds can germinate after 45 months of storage at room conditions (Debeaujon et al., 2000). Germination tests with mutant seeds during storage at room conditions showed that *lec1-3* and *abi3-5* seeds died within three months, independent of their viability at harvest. Nevertheless, cryo-storage at low temperatures (-80°C) significantly improved their survival.

Seed longevity strongly depends on the RH and T during storage. As expected, seed storage at high T and RH accelerated seed deterioration. The storage conditions were optimized to highlight differences in longevity for poorly storable mutant seeds. Conditions of storage of high T and RH that could accelerate aging were provided with the Controlled Deterioration Treatment (CDT), which also emphasized the variation in longevity between wild-type seeds from Landsberg *erecta* (L*er*) and Shahdara (Sha) accessions. CDT can reduce seed viability within a few days. At high T and RH the seed tissues get hydrated and cellular metabolism reactions which lead to the production of reactive oxygen species (ROS) are enhanced. This could result in a type of damage that is different from the progressive and slower tissue deterioration that occurs during long term dry storage.

4.1.3 The seed testa

The seed coat provides protection to the embryo (Haughn and Chaudhury, 2005). Natural aging of various Arabidopsis testa mutants, including *tt5-1*, showed that they have reduced longevity compared to the wild-type (Debeaujon et al., 2000). The experiments described in paragraph 3.2.6, in contrast, indicated that the *tt5-1* mutation can positively influence longevity. Furthermore, *tt5-1* mutant seeds germinated consistently better than L*er* seeds after CDT. The testa might therefore limit germination of seeds that undergo a rapid deterioration. The viable embryos, damaged either by the *abi3-5* mutation or by high T and RH conditions of the CDT, can probably break through the very thin seed coat of *tt5-1* mutants easier than through the L*er* seed coat.

4.1.4 Oxidative damage

Seed tissues accumulate oxidative damage during aging. ROS are generated by the spontaneous reactions of oxygen with cellular macromolecules and by imbalanced metabolic activities that occur during storage (Black et al., 2006). In the experiments described in this thesis, a direct correlation was observed between the level of oxidative damage on lipids and the longevity of seed batches in an *abi3-5* background. Furthermore, the level of oxidation increased during storage (data not shown). A direct correlation between the peroxidation level and the chlorophyll concentration was observed in *abi3-5* seeds (paragraph 3.2.4), thus suggesting that the pigments of inactive or uncoupled photosystems might catalyze the formation of oxygen radicals. In accordance with this, previous studies have shown that high amounts of chlorophyll in *Brassica oleracea* seeds correlate with reduced longevity (Jalink et al., 1998). The lipid peroxide level in *lec1-3* seeds was higher than in L*er*, but lower then in the seeds from the modifier line with the highest survival capacity, *lec*/Sha1/Sha2 (Figure 3.2.5). Anthocyanins, which are potent antioxidant molecules that accumulate in the *lec1* embryos (Meinke et al., 1994), might be less abundant in *lec*/Sha1/Sha2 embryos and could interfere in the chemical assays for the quantification of the lipid peroxides. Alternatively, the peroxide level in *lec*/Sha1/Sha2 seeds could be increased because they contained a larger proportion of lipids compared to *lec1-3*. This aspect was not investigated, but the modifier seeds with both Sha introgressions were about 50 % heavier than *lec1-3* mutants and contained significantly higher amounts of seed storage proteins (SSPs), which suggested that in *lec*/Sha1/Sha2 the maturation programs were partially restored. It is likely that also the storage lipids concentration was higher than in *lec1-3*, which could result in the accumulation of more peroxides. In this specific case, because of the dramatic differences in seed physiology between *lec1-3* and the *lec*/Sha modifiers, it was not possible to establish a direct negative correlation between lipid peroxidation and seed deterioration.

4.2 Proteomic analysis of seed deterioration in the *lec1-3* and *abi3-5* mutants

The most abundant Arabidopsis SSPs, the 12S globulins (cruciferins), are synthesized and processed to form the storage protein bodies during seed maturation. Other proteins dedicated to the protection of the embryo are also accumulated and provide tolerance to

desiccation and ROS damage. Late embryo abundant proteins (LEAs) and heat shock proteins (HSPs) protect membranes, proteins and nucleic acids from the deleterious removal of water. HSPs and LEAs are of greatest importance for seed longevity.

The proteomes of *lec1-3* and *abi3-5* seeds were characterized by a reduced abundance of seed storage globulins and their precursors, compared to L*er*. Furthermore they lacked of several types of LEAs and HSPs (See appendix, Table 3.1.4 and 3.1.5). For example, the HSP17.4, a protein that was correlated with desiccation tolerance in earlier studies (Wehmeyer and Vierling, 2000), was not detected in the *lec1-3* and *abi3-5* seed proteomes. The expression of HSP17.4 and other HSPs during seed maturation is mediated in particular by ABI3 (Kotak et al., 2007), but other transcription factors can also activate their expression in response to stress. This may be the reason why in *lec1-3* seeds, in which a strong metabolic response to stress is indicated by the accumulation of anthocyanins, many HSPs were abundant. The HSP identified by spot 86 for instance, was even increased compared to the wild-type, thus indicating that it has a general role in stress protection and that LEC1 is dispensable for its expression.

The *lec1-3* mutant seeds showed a more dramatic decrease of 12 S globulins and LEAs than *abi3-5* mutant seeds (Figure 3.1.14). This is in agreement with the fact that the LEC1 transcription factor acts upstream of the main genetic network of seed maturation (To et al., 2006). Consistent with that, *lec1-3* seeds generally deteriorated faster than *abi3-5* seeds.

The low abundance of LEAs and some HSPs proteins in *lec1-3* and *abi3-5* seeds is likely to be one reason of their poor longevity.

The proteome analysis revealed that proteins related to seed germination, such as the enzymes involved in the glyoxylate cycle (malate dehydrogenase, aconitate hydratase; Figure 3.1.13 and 3.1.14) and photosynthesis (rubisco precursors) were increased in both mutants, providing molecular evidence for their non-dormant phenotype. This aspect might also account for deterioration, since the maintenance of an active metabolism in dry seeds will enhance oxidative damage.

Seeds from the *abi3-5* mutant were specifically characterized by increased abundance of isocitrate lyase, an enzyme that plays a crucial role in the lipid mobilization via the glyoxylate cycle and in the synthesis of carbohydrates during seed germination and seedling establishment (Eastmond and Graham, 2001).

In *lec1-3* seeds, many germination and energy metabolism related proteins like the cell division cycle protein CDC48 and the enzyme phosphoenolpyruvate carboxykinase of gluconeogenesis (Penfield et al., 2004) were strongly increased, in accordance with the vivipary phenotypes that are often associated with *lec1* mutants.

Jasmonate (JA) hormone inducible proteins were more abundant in *lec1-3* than in L*er* (Table 3.1.5). The synthesis of JA can be a direct consequence of membrane damage (Creelman and Mullet, 1997). Accordingly, previous studies have shown that *lec1-3* mutant seeds are characterized by electrolytes leakage upon imbibition (Clerkx, 2004c), a symptom of membrane damage, which may inherently be an important component of the deteriorative damage in *lec1-3* seeds.

A common feature of *lec1-3* and *abi3-5* was the increased abundance (compared to L*er*) of two enzymes related to stress response and cell detoxification from ROS, namely the glutathione-dependent dehydroascorbate reductase and type 2 peroxiredoxin. The expression of these enzymes is probably induced in response to the seed deterioration processes of oxidative nature in the mutants seeds.

4.3 Modifiers of *lec1-3* and *abi3-5*

Natural accessions are a source of genetic variation for seed quality traits. The selection of *lec1-3* and *abi3-5* natural modifiers described in this work yielded three near isogenic lines (NILs) that showed improved longevity compared to the mutant parents. Due to incomplete penetrance and the possible presence of additional modifiers in the introgression lines, fine mapping was not possible. However, the *lec*/Sha2, *lec*/Sha1 and *abi*/Sei modifiers could be roughly mapped (paragraph 3.1.2). A more accurate mapping will be possible by the analysis of several biological replicates of NILs with introgressions of Sha and Seis am Schlern (Sei) that overlap within the regions of the modifiers in a *lec1-3* or *abi3-5* mutant background.

Natural variation for other traits with more general implications on plant development, such as flowering time, can also influence longevity. During the selection of the *lec*/Sha modifier lines, the F2 progenies with the highest longevity were selected after each backcross with L*er*. It is remarkable that after six backcrosses both *lec*/Sha lines still contained a Sha introgression in the region of 1.6 Mb at the top of chromosome four. The

flowering time gene *FRIGIDA* (*FRI*) is located within that region (Johanson et al., 2000). Lines with the Sha allele for *FRI* flowered and reached senescence slightly later compared to those with the early flowering L*er* allele (including the *lec1-3* mutant). At harvest the deterioration processes in the seeds of the early flowering plants were more advanced. Therefore, in a desiccation sensitive background, such as *lec1-3*, alleles of genes with minor effects on seed physiology, such as *FRI*, can be selected as enhancers of longevity. This also applies to *abi3-5*, as demonstrated by the difficulties encountered during the mapping of the mutant suppressor modifiers. Besides *sua3*, all the suppressor modifiers showed an increase of longevity in *abi3-5* background, but the phenotypes of *sua1* and *sua2* were too weak for mapping. In the mapping populations of these mutants, the segregation of allelic variation between the parental lines most probably interfered with the recognition of the *sua* mutant phenotypes.

4.4 The physiology of the natural modifiers highlights specific aspects of longevity

The *abi*/Sei modifier line showed a higher germination than *abi3-5* at all times after harvest, but the rate by which its seed viability decreased over time did not differ. This indicates that the beneficial effects on longevity of the *abi*/Sei modifier are restricted to the pre-harvest phase, when the modifier could slow-down the desiccation damage during the drying phase. This hypothesis was supported by the analysis of sorption moisture isoterms which indicated that *abi*/Sei seeds have an increased capacity of absorbing the moisture from the environment compared to L*er* and *abi3-5* (data not shown). A more detailed analysis on the *abi*/Sei seed properties, like the abundance and composition of non reducing sugars, could underline specific differences in the capacity to retain moisture during desiccation. Analysis of the seed proteome profile showed only minimal differences between *abi*/Sei and *abi3-5*. In addition, a CDT on the wild-type parental lines L*er* and Sei, showed that they respond very similar to artificial aging. Therefore, the effects of Sei alleles on longevity might affect specific aspects of the *abi3-5* mutant phenotypes.

The *lec*/Sha modifier seeds survived storage substantially better than *lec1-3*. This improvement was correlated with altered features of the seed, such as increased weight and protein composition. The seeds from the lines *lec*/Sha1 and *lec*/Sha2 were significantly heavier than *lec1-3* mutant seeds and also showed dormancy, while *lec1-3* seeds in the L*er*

background often germinate precociously. Furthermore, the line with both Sha introgressions (*lec*/Sha1/Sha2) produced seeds with the highest longevity, weight and the strongest dormancy, indicating that *lec*/Sha1 and *lec*/Sha2 are independent modifiers with additive effects. Notably, the *lec*/Sha1/Sha2 seeds were even heavier than the L*er* control ones. Seeds from the Sha accession were both heavier and more resistant to artificial aging than L*er* and suggested the presence of alleles that influence seed weight, which could co-locate with the Sha modifiers. Previous work on one L*er*/Sha RIL population identified QTLs for seed weight and dormancy at the bottom of chromosome 5, which coincides to the map position of *lec*/Sha2, the modifier with the strongest effect. The Sha allele was found to increase both traits (Clerkx 2004b, El-Lithy et al., 2004). A CDT of four lines with Sha introgressions in L*er* background in the same region revealed the presence of a minor QTL for seed longevity, which overlaps with the QTLs for dormancy and seed weight previously identified (paragraph 3.1.7). This result suggests that seeds with dormant embryos are less sensitive to deterioration reactions because their cellular metabolism is more reduced during storage. However it could also mean that the QTLs found in the L*er*/Sha populations are not identical but genetically linked. In fact, a previous analysis for seed storability using a L*er*/Cvi RIL population (Bentsink et al. 2000) did not identify any QTL for seed longevity in the region of *DELAY OF GERMINATION 1* (*DOG1*) gene, which is a major QTL for seed dormancy in Arabidopsis (Bentsink et al., 2006).

The substantial changes in the seed physiology of the *lec*/Sha lines, including those resulting in dormancy, might originate from a specific interaction between the Sha alleles and the *lec1-3* mutant. An analysis of the seed proteome of the *lec*/Sha modifiers revealed that they had an increased abundance of SSPs, compared to *lec1-3*. The 12S storage proteins were more abundant in *lec*/Sha2 than in *lec*/Sha1 and the line containing both Sha introgressions showed the highest levels, indicating that the *lec*/Sha1 and *lec*/Sha2 modifiers may act additively as regulators of the same pathway. The abundance of storage proteins was correlated with longevity and dormancy phenotypes. It was previously shown that the 12S globulins belong to the most oxidized proteins in dry seeds (Job et al., 2005), therefore, it is possible that an increased abundance of SSPs provide a buffer to the oxidative reactions that damage the embryo cellular structures. Radical reactions to the detriment of SSPs are probably less critical for the success of germination than the impairment of membranes or DNA. Apart from the 12S globulins, close to wild-type levels

of LEAs and HSPs were also detected in the *lec*/Sha modifiers seeds. However, the reversion of *lec1-3* effects on the seed proteome was not complete. Proteins specific for *lec1-3*, but not for *abi3-5*, like jasmonate inducible proteins, were maintained in the *lec*/Sha seeds (see as an example in Figure 3.1.16). Consistent with that, some of the pleiotropic effects characteristic for *lec1-3*, such as cotyledon trichomes and high levels of anthocyanins in the embryo, were also maintained in *lec*/Sha seeds. The *lec*/Sha modifiers can restore the accumulation of storage proteins, and therefore activate a specific subset of the LEC1-controlled genes responsible for seed maturation. LEC1 controls the expression of the SSP genes in a hierarchical manner, which involves ABI3 and FUS3 transcription factors (Kagaya et al., 2005). Both FUS3 and ABI3 can bind to a cis-element, called the RY repeat (CATGCA), which is present in the promoter of SSP genes and activate their expression in a partially redundant manner. Consistent with this, the seed proteome of *lec1-3* showed a more severe reduction in SSP abundance compared to *abi3-5*. Besides cooperatively participating in the activation of SSP, FUS3 and ABI3 have also distinct, specific targets. For example FUS3 activates the expression of an aspartic proteinase (AP), an enzyme necessary for the maturation and catabolism of SSPs, which is not regulated by ABI3 (Kagaya et al., 2005). The AP protein was identified in the *abi3-5* mutant seed proteome, which suggests that FUS3 is maintained in *abi3* mutants, but was almost undetectable in *lec1-3* seeds, thus confirming the findings of Kagaya et al. (2005). Interestingly, in the proteome profile of the *lec*/Sha seeds the AP protein was also detected, indicating that, despite the absence of LEC1, specific functions of the FUS3 transcription factor could be restored (Figure 3.1.17).

Another distinctive aspect of *lec*/Sha modifiers seeds is dormancy. LEC1 is a key player of the hierarchical regulatory network that controls seed maturation and probably regulates dormancy indirectly. ABI3 is essential for the ABA mediated dormancy and has an important role in mediating the inhibition of germination in response to both ABA and sugars. The *lec1* mutants have reduced ABA sensitivity, probably because of a decreased accumulation of ABI3. Germination of *lec*/Sha seeds, particularly *lec*/Sha2, was inhibited on media containing 10% sucrose (data not shown), thus indicating that the dormancy phenotypes of the *lec*/Sha lines might be due to the restored expression of *ABI3*. In the original natural modifiers selection breeding, 20 Arabidopsis accessions were crossed to both *lec1-3* and *abi3-5*, but the Sha modifiers were only identified in *lec1-3* background.

This suggests that the Sha modifiers are *lec1-3* specific and might not suppress the *abi3-5* phenotype.

LEC1 is homologous to the HAP3 subunit of a trimeric CCAAT-binding factor that is widely distributed from fungi to mammals (Lotan et al. 1998). LEC1 may function in a similar way in Arabidopsis, as the subunit of a complex that regulates a specific set of genes mainly related to embryo development, including *ABI3* and *FUS3*. Although the downstream genes of LEC1 are known, neither direct targets nor the *cis*-elements of the putative LEC1 complex have been identified up to now.

The Sha modifiers are likely to be positive regulators of *ABI3* and *FUS3* that act independently from LEC1. They might be allelic variants of regulatory elements downstream of the LEC1 complex. The identification of such alleles could lead to a better understanding of the molecular mechanism of LEC1 action in the initiation of seed maturation programs.

Mapping approaches conducted so far could not narrow the map position of the *lec*/Sha modifiers accurately, but fine-mapping could be successful by using the accumulated knowledge on their physiology. For example, the progeny seeds of populations segregating for *lec*/Sha in *lec1-3* background could be tested for ABA sensitivity at germination, whereby higher sensitivity would indicate the presence of the Sha modifiers.

4.5 Alterations in the RNA metabolism can influence ABI3

RNA metabolism plays a crucial role in the regulation of gene expression during all stages of plant growth, including seed development. This regulation is achieved by RNA-binding proteins (RBPs). In addition to complex genetic interactions with *LEC1* and *FUS3*, the *ABI3* gene expression is controlled at the post-transcriptional level. For example, the long 5′ untranslated region of the *ABI3* gene has been proposed to negatively regulate *ABI3* expression via a post-transcriptional mechanism (Ng et al., 2004). In addition, alternative splicing of *ABI3* homologues was detected in wheat and other cereal species (McKibbin et al., 2002).

The strongest of the suppressor modifier mutations, identified as *sua4* and renamed *sua-1*, reverted all of the *abi3-5* mutant phenotypes including poor seed longevity. Immunological analysis revealed that, *abi3-5* seeds contain a truncated form of the ABI3

protein (ABI3-5 protein). This protein was restored to a nearly full-length size in the *sua-1 abi3-5* double mutant, which explained the phenotypes of this mutant-suppressor line. The *sua-1* mutation caused the splicing of an intron-like sequence (cryptic intron) from the *abi3-5* mRNA transcript. This 77 bp long cryptic intron is located 57 bp downstream of the single nucleotide deletion of *abi3-5* and its removal from the mutant transcript restores the reading frame. The translated polypeptide coded by the novel splice variant (the ABI3-5SV protein) differs from wild-type ABI3 by 21 erroneous and 26 deleted codons, but maintains most of its molecular functions, as demonstrated by the *sua-1 abi3-5* seed phenotypes. None of the ABI3 functional domains is directly affected by the changes in the ABI3-5SV protein sequence. In the *sua* mutant background the cryptic intron is also spliced out in wild-type *ABI3* plants and in other *abi3* mutants, but there it leads to non-functional truncated ABI3 proteins.

The portion of the *abi3-5* transcripts that is abnormally spliced in *sua-1 abi3-5* developing seeds appears to be relatively small, based on RT-PCR assays. Nonetheless, in *sua-1 abi3-5* double mutant seeds the novel splice variant is sufficient to accumulate detectable amounts of ABI3-5SV protein.

The cryptic intron has sequence similarities with canonical plant introns, particularly the consensus sequences at the two borders (Figure 3.3.12), but it lacks a polypirimidine stretch in the 3` end that generally characterizes introns (Reddy, 2007). These intron defining motifs present on pre-mRNAs are recognized and bound by specific small ribonucleoprotein particles. Subsequent formation of a large complex of proteins (the spliceosome) catalyzes the excision of the intron.

The cryptic intron of *ABI3* might be left-over of an ancient intron that is not recognized anymore by the splicing machinery in the wild-type plants. This could be verified by comparative analysis of *ABI3* homologues from other species. Alternatively, the sequences similarity with an intron could be a random event. In any case, the *sua-1* mutant appears to influence the specificity of splicing site recognition, which in this case led to splice variants that resulted in functional proteins.

4.6 The SUA protein has features of spliceosomal proteins

Analysis of the SUA protein sequence revealed that its conserved domain architecture, that includes two RNA recognition motifs (RRMs), is characteristic for proteins involved in RNA metabolism. The RRM domain is the most widespread eukaryotic RNA binding module and mediates RNA recognition in hundreds of proteins belonging to the RNA processing machinery (Burd and Dreyfuss, 1994). The SUA protein shares a high sequence similarity with the human tumor suppressor gene *hsRBM5* (see paragraph 3.3.1), which has been proposed to regulate alternative splicing of apoptotic genes (Oh et al., 2006). Similarly to what has been observed for tumor cells overexpressing *hsRBM5*, also the ectopic expression of *SUA* probably causes lethality in Arabidopsis.

The experiments conducted so far indicated that the cellular abundance of *SUA* transcripts is controlled at the post-transcriptional level, similar to many genes of spliceosomal proteins. For instance, the serine/arginine-rich protein transcripts are consistently alternatively spliced in a developmental and tissue-specific manner (Isshiki et al., 2006). Three splice variants of the *SUA* gene were identified, two of them code for truncated proteins. In the *sua-1* mutant the two variants with premature stop codons showed a reduced abundance (Figure 3.3.2), which indicates that the SUA protein could be involved in its own alternative splicing.

Another similarity between SUA and other splicing related proteins is their cellular localization. The fluorescence patterns observed inside the nuclei transformed with the chimeric *SUA_GFP* gene showed speckles of fluorescence, similar to those obtained with serine/arginine-rich_GFP proteins (Ali et al., 2003). The microscopy observations also indicated that *SUA* expression might be tightly controlled, because not all the nuclei of stably transformed *SUA_GFP* plants showed a detectable fluorescence signal.

Based on its functional motifs, SUA might bind directly to specific RNA targets. However, SUA could also be part of a complex like the spliceosome, which in Arabidopsis is composed of about 300 proteins (Reddy et al., 2007), and interact with the mRNA targets indirectly.

The *sua-1* mutant showed a seed specific phenotype, which include reduced longevity and increased sensitivity to ABA. The *sua-2* and *sua-3* single mutant seeds instead, did not show any phenotypic difference compared to wild-type Columbia (Col). This discrepancy could be explained by the effects of modifiers of the *sua* mutations present in the Col

genetic background. It is also possible that *sua-1*, which is the only *sua* allele transcribed of those studied, is translated into a truncated protein (SUA-1). This polypeptide would include the RRMs and the Zn finger motifs but would lack the G patch domain at the carboxy terminus. The putative SUA-1 protein might compete for substrates with the mRNA processing machinery and therefore function as a dominant negative allele. This hypothesis implies that *sua-1* heterozygous seeds should also display seed specific phenotypes similar to the *sua-1* homozygous seeds, but so far this was not observed. The possibility that SUA binds transcripts of genes involved in seed maturation must be investigated. Furthermore, in order to understand if SUA is a member of the spliceosome, the identification of SUA partner proteins is necessary. Experiments for the immunoprecipitation of tagged SUA from total extracts of developing seeds might help to clarify these issues.

Considering the specificity of the suppression effects studied in *sua-1 abi3-5* double mutant, SUA does not seem to affect seed longevity specifically, but rather has a more general role on RNA metabolism.

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Appendix

Table 3.1.4: Identified proteins with increased (red) or decreased (blue) abundance in *abi3-5* freshly harvested seeds, compared to wild-type L*er* dry mature seeds

Spot number	Protein name	AGI N°	Exp MW	Exp IP
1	Ribulose bisphosphate carboxylase large chain [precursor]	AtCg00490	57.36	5.77
2	Malate synthase	At5g03860	62.96	8.00
3	Malate synthase	At5g03860	62.91	7.08
4	Malate dehydrogenase, glyoxysomal	At2g22780	38.31	7.42
5	Formate dehydrogenase	At5g14780	41.09	6.09
6	40S ribosomal protein SA	At1g72370	38.55	5.02
7	Glutathione-dependent dehydroascorbate reductase	At1g19570	25.34	5.43
8	Peptidase M1 family protein,	At1g63770	96.42	5.43
9	Pyruvate,orthophosphate dikinase	At4g15530	96.35	5.37
10	Ribulose bisphosphate carboxylase large chain [precursor]	AtCg00490	57.36	5.57
11	Ribulose bisphosphate carboxylase large chain [precursor]	AtCg00490	57.36	5.82
12	Isocitrate lyase	At3g21720	62.77	6.30
13	D-fructose-1,6-bisphosphate 1-phosphohydrolase	At1g43670	40.92	5.29
80	Peroxiredoxin type 2	At1g65980	16.14	5.18
15	HSP 17.4	At3g46230	17.93	4.99
16	HSP 17.7	At5g12030	17.94	5.50
17	Beta-cruciferin 12S (seed storage protein precursor fragment)	At1g03880	16.14	5.71
18	Beta-cruciferin 12S (seed storage protein precursor fragment)	At4g28520	23.32	6.89
19	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	At3g04120	40.29	6.49
20	HSP 70	At1g16030	76.38	5.24
21	Jasmonate inducible protein-like	At3g16460	77.81	5.29
22	Late embryogenesis abundant (LEA)	At3g22500	34.02	5.36
23	Late embryogenesis abundant (LEA)	At2g42560	66.68	5.74
24	Late embryogenesis abundant (LEA)	At2g42560	66.68	5.78
25	Elongation factor 1-beta-gamma	At1g09640	41.75	5.66
26	12S seed storage protein [precursor]	At5g44120	49.43	7.19
27	12S seed storage protein [precursor]	At5g44120	50.44	7.67
28	Alpha-globulin 12S (seed storage protein fragment)	At4g28520	23.94	5.58
29	Alpha-globulin 12S (seed storage protein fragment)	At4g28520	25.42	5.78
30	Alpha-globulin 12S (seed storage protein fragment)	At4g28520	32.64	5.85
31	Alpha-globulin 12S (seed storage protein fragment)	At1g03880	27.20	6.50
32	Cupin family protein Beta subunit	At1g03890	18.84	5.68
33	Beta-cruciferin 12S (seed storage protein precursor fragment)	At1g03880	15.80	5.72
34	Late embryogenesis abundant (LEA)	At3g53040	61.88	5.06
35	Late embryogenesis abundant (LEA)	At3g17520	34.92	5.21
36	Glycosyl hydrolase family 1 protein	At3g21370	64.62	6.08

37	Phosphatidylethanolamine-binding family protein	At5g01300	17.82	5.10
38	Late embryogenesis abundant (LEA)	At2g36640	60.51	5.22
39	12S seed storage protein [precursor]	At4g28520	56.89	7.19
40	Short-chain dehydrogenase/reductase family protein	At5g50600	40.31	5.88
41	Alpha-globulin 12S (seed storage protein fragment)	At1g03880	25.42	5.78
42	Aldose reductase	At5g01670	38.48	6.22
43	Alpha-globulin 12S (seed storage protein fragment)	At4g28520	23.54	5.33
44	Glycosyl hydrolase family 1 protein	At3g21370	64.96	5.92
45	Glycosyl hydrolase family 1 protein	At3g21370	65.26	6.08
46	Cupin family protein	At2g28490	27.24	5.70
47	Alpha-globulin 12S (seed storage protein fragment)	At4g28520	34.11	5.87
48	Superoxide dismutase (Mn)	At3g56350	24.39	6.18
49	Beta-cruciferin 12S (seed storage protein precursor fragment)	At1g03880	18.43	5.85
50	Alpha-globulin 12S (Seed storage protein fragment)	At5g44120	14.51	5.86
51	Low-temperature-induced 65 kDa protein or Desiccation-responsive protein 29B	At5g52300	92.73	4.96
52	Low-temperature-induced 65 kDa protein or Desiccation-responsive protein 29B	At5g52300	93.01	4.94
53	Heat shock protein 17.6A	At5g12030	17.08	5.51
54	Glutathione peroxidase	At4g11600	18.27	5.83
55	Pathogenesis-related protein Bet v I family	At1g70840	16.88	6.30
57	Cupin family protein alpha subunit	At2g28490	29.55	5.22
93	Beta-cruciferin 12S (seed storage protein precursor fragment)	At1g03880	18.43	6.36
99	Cupin family protein Beta subunit	At1g03890	17.59	5.68

Table 3.1.5: identified proteins with increased (red) or decreased (blue) abundance in *lec1-3* freshly harvested seeds, compared to wild-type L*er* dry mature seeds

Spot number	Protein name	AGI N°	Exp MW	Exp PI
7	Glutathione-dependent dehydroascorbate reductase	At1g19570	25.34	5.43
8	Peptidase M1 family protein,	At1g63770	96.42	5.43
9	Pyruvate,orthophosphate dikinase	At4g15530	96.35	5.37
10	Ribulose bisphosphate carboxylase large chain [precursor] or rubisco	AtCg00490	57.36	5.57
11	Ribulose bisphosphate carboxylase large chain [precursor] or rubisco	AtCg00490	57.36	5.82
14	Myrosinase-associated protein	At1g54010	41.12	7.19
58	Aconitate hydratase cytoplasmic	At2g05710	96.38	5.75
59	Cell division cycle protein (CDC48)	At3g09840 or At5g03340	96.32	5.21
60	Translation elongation factor Tu (chloroplast) (without transit peptide)	At4g20360	42.94	5.43
61	Jasmonate inducible protein-like	At3g16420	36.28	5.49
62	Jasmonate inducible protein-like	At1g52110	62.82	6.33
63	Glycosyl hydrolase family 1 protein	At3g09260	63.47	6.56

64	5-methyl-tetra-hydropteroyl-tri-glutamate-homocysteine methyltransferase	At5g17920	82.27	5.94
65	Jasmonate inducible protein-like	At3g16420	64.75	5.66
66	DNA topoisomerase I	At5g55300	96.47	5.78
67	5-methyl-tetra-hydropteroyl-tri-glutamate-homocysteine methyltransferase	At5g17920	82.05	6.09
68	Ferritin 1, chloroplast [precursor]	At5g01600	25.34	5.19
69	Ribulose bisphosphate carboxylase large chain (Cterm)	AtCg00490	15.84	6.5
70	Glutathione S-transferase	At1g78370	25.34	5.6
71	Peptidase M1 family protein,	At1g63770	96.69	5.61
72	Peptidase M1 family protein,	At1g63770	96.65	5.54
74	Cell division cycle protein (CDC48)	At3g09840	97.41	5.2
75	Lectin family protein	At3g16450	35.03	5.05
76	10-formyltetrahydrofolate synthetase	At1g50480	76.41	6.22
78	Shepherd protein	At4g24190	96.35	4.58
79	26S proteasome regulatory subunit S5A (RPN10), identical to multiubiquitin chain binding protein (MBP1)	At4g38630	56.46	4
80	Peroxiredoxin type 2	At1g65980	16.14	5.18
81	20 kDa chaperonin, chloroplast (CPN21)	At5g20720	25.42	5.19
82	14-3-3 protein GF14 psi (GRF3) (RCI1)	At5g38480	32.64	4.8
83	Glycine dehydrogenase	At2g26080	97.42	5.72
84	Phosphoenolpyruvate carboxykinase (ATP)	At4g37870	72.97	6.44
85	Cell division cycle protein (CDC48)	At3g09840 or At5g03340 or At3g53230	96.47	5.09
86	Heat shock protein	At1g79930	101.44	5.04
87	Subtilisin-like protease	At5g67360	80.48	7.39
88	ATP-dependent Clp protease ATP-binding subunit / ClpC	At5g50920	94.01	5.59
15	HSP 17.4	At3g46230	17.93	4.99
17	Beta-cruciferin 12S (seed storage protein precursor fragment)	At1g03880	16.14	5.71
18	Beta-cruciferin 12S (seed storage protein precursor fragment)	At4g28520	23.32	6.89
22	Late embryogenesis abundant (LEA)	At3g22500	34.02	5.36
23	Late embryogenesis abundant (LEA)	At2g42560	66.68	5.74
24	Late embryogenesis abundant (LEA)	At2g42560	66.68	5.78
26	12S seed storage protein [precursor]	At5g44120	49.43	7.19
27	12S seed storage protein [precursor]	At5g44120	50.44	7.67
28	Alpha-globulin 12S (seed storage protein fragment)	At4g28520	23.94	5.58
29	Alpha-globulin 12S (seed storage protein fragment)	At4g28520	25.42	5.78
31	Alpha-globulin 12S (seed storage protein fragment)	At1g03880	27.2	6.5
32	Cupin family protein Beta subunit	At1g03890	18.84	5.68
33	Beta-cruciferin 12S (seed storage protein precursor fragment)	At1g03880	15.8	5.72
35	Late embryogenesis abundant (LEA)	At3g17520	34.92	5.21
37	Phosphatidylethanolamine-binding family protein	At5g01300	17.82	5.10

40	Short-chain dehydrogenase/reductase family protein	At5g50600 or At5g50700	40.31	5.88
47	Alpha-globulin 12S (seed storage protein fragment)	At4g28520	34.11	5.87
49	Beta-cruciferin 12S (seed storage protein precursor fragment)	At1g03880	18.43	5.85
89	Beta-cruciferin 12S (seed storage protein precursor fragment)	At5g44120	14.86	5.87
90	Cupin family protein Alpha subunit	At1g03890	27.24	5.20
91	Alpha-globulin 12S (seed storage protein fragment)	At4g28520	33.89	6.24
92	Alpha-globulin 12S (seed storage protein fragment)	At4g28520	34.35	6.42
93	Beta-cruciferin 12S (seed storage protein precursor fragment)	At1g03880	18.43	6.36
94	Beta-cruciferin 12S (seed storage protein fragment)	At1g03880	18.08	6.12
95	Malate oxidoreductase or malic enzyme	At2g19900	64.79	6.35
96	Aspartic proteinase (precursor)	At1g62290	32.51	5.76
97	Large subunit aspartic proteinase	At1g11910	28.69	5.06
98	Glycosyl hydrolase family 35 protein	At4g36360	65.24	6.02
99	Cupin family protein Beta subunit	At1g03890	17.59	5.68
100	Late embryogenesis abundant (LEA)	At2g42560	66.68	5.79

Table 3.2.1: List of polymorphic markers used for the fine mapping of the *sua-1* mutation

Marker name	Marker type	Physical position (chromosome 3)	Forward primer sequence	Reverse primer sequence
F24M12	SSCP	19,178	TTGGGATTACTTTGCCAG	CGATGTAAACCCAGTGATG
F8J3	SSLP	19,598	GAACAGCCAATTGAAGCAAGC	AAAAATGTTGATCAGAGCTCAAGTG
F8J2	CAPS	19,627	GACTAAGGTGGAGAGGTTG	GGGAGGTGTAAGAGAACTG
T4D4	SSLP	19,690	TGACGTCATAAAGAACTGGTTGG	TTTGCGATTCAGGTCCATTG
F4P15	SSLP	19,803	CGTCGAGACTTTACTCGATTTTGC	CGGTGAAGACGACGAAGATG
F4P14	SSLP	19,900	CGAAACCTTTCCGATGGATG	TTTCTTCTCTGCCGCTTTCG
F4P12	SSLP	19,909	TATTTAGCGGGAGAACTG	TACTTAATTCCAGCCACAG
F5K20	SSLP	19,909	CTAGTGTAACGTGTTGCGTTGG	CTTATTATTTTAAACAGAGCCGATCC
F5K23	SSCP	19,930	CAATCAACAGAATACTCCAG	TAAGTATTTCTCTAGGGTATCC
F5K21	SSLP	19,938	CCGAGTCTTTTGCTTAATTTGC	CAAATGCCAAATGGTATGTAGG
F5K24	CAPS	19,998	GCATCATCTCTTATTGCTTCGTG	TCAGTGTGATTGTTGTTATTGAACC
539	CAPS	19,999	GGATTATGCTGAGGAAAG	GTGACAATACAAGCTACAATC
F24B22	SSLP	20,026	CCTATAGCCGCATGGAGAAGAC	TACACACACACCCCACAACATC
F24B25	CAPS	20,030	CAGTTTGAGTGGGTTGATTTGG	ACTTCTCCATCTGGGTCTGAGC
F24B26	CAPS	20,048	AAAAATCGCCACCGAGATATTG	CTGAATTGTTTGAGGGTTTTGTTG
F24B27	CAPS	20,056	AGACAACCTCCAAGAAGCCAAG	GCATGGGAAATCTTAACAAAAAGC
F24B28	CAPS	20,072	TCTCAAGTGAATGTAAAACACAATGG	TGAAAGACCCTTTATTCCACCTG
F24B24	CAPS	20,084	GTTAAAGTTGGATTCGGCCAAG	AGCCGATAACCAGAGGTCAGAG
542	CAPS	20,100	TCGGTTTCTCCACTCCAG	AGATGGCGTTGTGGAGAG
1149	CAPS	20,115	TCCTTTCTGATCCTTCAGTTGG	TAACGACTCCGATGTCATCAAC
F24B23	SSLP	20,115	TGTTTTGTCGGTGTTCAAGTCC	TGAAAACAATTGCCAATCATGG
1201	CAPS	20,120	CCATAAAGAAAATTGCTGATCG	TTCTTTCCAGACCTTTGTCAAC
1266	CAPS	20,127	TCTTAAACCACTCTGCTTCCTC	CTCTCTGCATTTCTTCCAATTC

T12E18	CAPS	20,133	GGCGTTTGTAAACGTCAAATCAG	AGGCAAATCCGGGAAATTATTG
T12E19	CAPS	20,158	AATCTCTGCTTGCTCGTTGTTG	CCCCTAATGTCTCGCTACTTCC
T14E15	CAPS	20,176	ATTATTCTCCGCCACTTTGAGC	GAGCTCTCTGAAGTGATGGAAGC
545	CAPS	20,187	TTATAATGGGAAAGGATGG	ATGAAGAGCACGAACTGAC
T14E13	CAPS	20,196	GGTCATTTAAGGCAAGATGCTG	AGCATTCTACGTATCGCAGTCG
T14E14	CAPS	20,196	GGTCATTTAAGGCAAGATGCTG	AGCATTCTACGTATCGCAGTCG
T14E12	CAPS	20,203	GCTAGAGCTCTGTTCGTGCAAC	ATATCCGCTTTCAAAACCATCG
T14E10	CAPS	20,231	AAGAAGCGGGAAATAAGAAAGC	GGCTTTGTTTTGGAGATGACAG
T5N23	SSLP	20,271	CAGTGGTTTCTTTGGCCTTTG	TCCCTATCTCTCACGATAGTGCTG
F28P11	SSLP	20,308	TTCTTCCGGCATATCTTCAGTG	GCCTTGACACCAGAAATCCTC
F28P10	CAPS	20,347	TCATTGGGAGATAAAGCCATTC	TGCATTTTCCCAGGGATGTC
T15C9	SSLP	20,405	GAATGAAGTTGAAGCAGGAGG	CTCAGTGTCAAGGAATCGAAAC
T15C10	SSLP	20,405	CGGTGAAGAGTTGGTGAGAGG	TCAAGGAATCGAAACGCAGTC

Table 3.3.1: List of molecular markers for the characterization of *SUA*

Marker name	**Use**	**Forward primer sequence**	**Reverse primer sequence**
abi3-5	CAPS marker for genotyping the *abi3-5* allele	GAATCTCCACCGTCATGGTCAC	ACAAGAACCTCCTCTGTCTCGC
sua4	SSLP for genotyping the *sua-1* allele	ATGGGCAAACTGAAATCTGATG	TCTATGAGTAGGCCCTGCAGTC
abi3174	CAPS for genotyping the *abi3-1*, *abi3-4* and *abi3-7* alleles	TGGAAACCAGAAAAGAATTTGC	CTAGCTCCGGCAAGTGTGTC
mabi351f-353r	detects the *abi3-5* splice variant	CCGGGTTTTGGATACATGC	GCAAGAGAAACCGCAAATTC
cEEL	RT-PCR marker	TGTCTCAGACCCACCACTTG	CCAGCTCTCAACAACAAATCC
cHSFA9	RT-PCR marker	TCAAGCACAAGAACTTCTCCAG	CTCAACCTCAGTCTCTGTCGTG
cABI5	RT-PCR marker	GTGAGACTGCGGCTAGACAAC	TTCCTCTTCCTCTCCAACTCC
cACT2	RT-PCR marker	GTATGGTGAAGGCTGGATTTGC	TGAGGTAATCAGTAAGGTCACGTCC
cSUA	*SUA* cDNA amplification	TCATGGATCCTAGTCGATATGG	GTCAATTATTGTCAGACATGTCACG
cSUAf-859	Sequencing of *SUA* cDNA	TCATGGATCCTAGTCGATATGG	TCCGACCTCTAGGAGATCTTG
857-876	Sequencing of *SUA* cDNA	AGTATGAAAGGGCCAGTGTGAG	GGACATGAGTTGGACCTGTCTC
865-897	Sequencing of *SUA* cDNA	GCATGATGGTATAGTTTTGGATGG	CCAAAGCCCACTATTGCTGTC
887-904	Sequencing of *SUA* cDNA	GTATATCAGCACCCTCGCACTC	TCTATGAGTAGGCCCTGCAGTC
902-cSUAr	Sequencing of *SUA* cDNA	ACCTCCAATCATGGAGTTTCTGCC	GTCAATTATTGTCAGACATGTCACG

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