Genetic analysis of seed development in *Arabidopsis thaliana*

Genetische analyse van de zaadontwikkeling in *Arabidopsis thaliana*

Promotor: dr. ir. M. Koornneef
persoonlijk hoogleraar
bij de vakgroep Erfelijkheidsleer

Karen Léon-Kloosterziel

Genetic analysis of seed development in *Arabidopsis thaliana*

Proefschrift

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Stellingen

(I) De genetische benadering in het onderzoek naar kiemrust laat zien dat de regulatie van kiemrust een complex proces is waarbij zeer veel genen betrokken zijn.
Dit proefschrift; Van der Schaar et al. (1997) Heredity 79:190-200

(II) De analyse van reciproke kruisingen en hun nakomelingschap is een efficiënte methode om vast te stellen of kiemrust bepaald wordt door het embryo of door de zaadhuid.
Dit proefschrift

(III) Door Meinke et al. en Parcy et al. wordt een verschil in ABA-gevoeligheid van *lec1* zaden gevonden. Dit verschil kan te wijten zijn aan de verschillende definities van kieming van deze auteurs.
Meinke et al. (1994) Plant Cell 6:1049-1064; Parcy et al. (1997) Plant Cell 9:1265-1277

(IV) Men dient voorzichtig te zijn met het interpreteren van ongevoeligheid voor gibberellineremmers als een verminderde gibberellinebehoefte.

(V) Het verdient aanbeveling om voor fysiologische experimenten in het onderzoek naar kiemrust bij *Arabidopsis* niet alleen gebruik te maken van de ecotypes L*er* en Col, maar ook van ecotypes met een diepere kiemrust, zoals Cvi.

(VI) Onkruid vergaat niet, behalve als het ABA-deficiënt is.

(VII) Het zou eerlijker zijn als in de procedure voor de acceptatie van artikelen voor wetenschappelijke tijdschriften niet alleen de referenten, maar ook de auteurs anoniem zouden zijn.

(VIII) Het in sommige genetica-leerboeken gebruikte voorbeeld van blauwe en bruine oogkleur als illustratie van de begrippen dominant en recessief gaat volkomen voorbij aan de complexiteit van de genetica van menselijke oogkleur.
Müntzing (1961) Genetics: basic and applied; Papazian (1967) Modern genetics

(IX) De effectiviteit van veel milieumaatregelen valt te betwijfelen, omdat men er ten onrechte van uit gaat dat hogere kosten, verbonden aan milieu-onvriendelijk gedrag, automatisch zullen leiden tot een vermindering van dit gedrag.

(X) Het is te hopen dat behalve de vaders tot wie de SIRE-campagne "Mannen zijn thuis net zo onmisbaar als op het werk" zich richt, ook hun werkgevers zich aangesproken zullen voelen.

(XI) De kwalificatie "heerlijk, helder" behoort niet voorbehouden te zijn aan bier.

Stellingen behorend bij het proefschrift "Genetic analysis of seed development in *Arabidopsis thaliana*" door Karen Léon-Kloosterziel, te verdedigen op 5 november 1997 te Wageningen

Abstract

This thesis deals with the genetic aspects of seed development in *Arabidopsis thaliana*. Mutants affected in several aspects of seed development and, more specifically, in seed maturation have been isolated by various selection procedures. The mutants have been analyzed genetically, physiologically, and morphologically. Some of the mutants are impaired in the biosynthesis or sensitivity to the plant hormone, abscisic acid (ABA). All ABA-related mutants show reduced seed dormancy, indicating the important role of this hormone in the establishment of dormancy. In a direct screen for reduced dormancy, two mutants (*rdo*) with reduced dormancy were found. These were not ABA-deficient and showed the same sensitivity to ABA, ethylene, auxin, and cytokinin as the wild-type. In contrast to this embryo-determined reduced dormancy, reduced dormancy can also originate in an altered seed coat (testa), like in the altered testa shape (*ats*) mutant. Here, the altered testa shape is caused by a defect in the development of the integuments. Extreme ABA-insensitive mutants (*abi3*) have green seeds that fail to complete many other aspects of seed maturation, including the induction of dormancy and desiccation tolerance, and the accumulation of seed storage proteins and lipids. In addition to *abi3* mutants, *lec* and *fus* mutants exhibit such a severely disturbed seed maturation as well, with dark purple seeds due to anthocyanin accumulation. The *fus3* mutant shows normal ABA-sensitivity. These various seed maturation mutants indicate that specific genes, some acting dependently and some acting independently from ABA, are responsible for seed maturation programs. The seed maturation mutants were subjected to a physiological and biochemical analysis. A GA-deficient mutant was combined with these mutants. Analysis of these double mutants indicated that seeds of the *abi3* and *lec* mutants did not require GA for germination, in contrast to *fus3* seeds. This correlates with ABA-sensitivity for germination. The composition of storage proteins and carbohydrates in *abi3*, *lec*, and *fus3* mutant seeds has been compared. The *abi3*, *lec*, and *fus3* mutants all showed severely reduced storage proteins. The desiccation intolerance of these seed maturation mutants was not correlated with the lack of specific carbohydrates. Furthermore, the mutants had a higher total content of carbohydrates. This is probably a consequence of the lower levels of storage lipids and proteins.

Contents

CHAPTER 1

General introduction

General introduction

Seeds represent a crucial stage in the plant's life cycle. It is an appropriate structure for dispersal and survival during less favourable periods. This thesis deals with the investigation of genetic aspects of seed development in a member of the Cruciferae family, *Arabidopsis thaliana.*

Arabidopsis is the model

During the last decade, *Arabidopsis* has become an important model species in plant research, because of several advantages: it is a self-pollinator that requires little space and has a short life cycle, making it very suitable for genetic research. It has a low amount of DNA, approximately 100 000 kb with few repetitive sequences; much international effort is being made to sequence the complete genome. There are elaborate linkage and physical maps available of the 5 chromosomes of *Arabidopsis* (Meyerowitz, 1989; Dean and Schmidt, 1995). *Arabidopsis* has a very wide distribution over the world. Many "ecotypes", which are pure lines due to the self-pollinator character of the species, are available for research. The lines Landsberg *erecta* (L*er*), Columbia (Col), and Wassilewskija (Ws) are mostly used for research.

Seed development in *Arabidopsis*

Seed development in *Arabidopsis* is a process that takes 2-3 weeks, dependent on growth conditions. First, a period of mainly morphological changes (embryogenesis) and after that a period of biochemical and physiological changes (maturation) can be distinguished (Meinke, 1994; Koornneef and Karssen, 1994). The seed develops from the ovule, which consists of the embryosac surrounded by the integuments (Reiser and Fisher, 1993). After fertilization of the embryosac, the formation of the embryo and the endosperm starts. At the same time, an elongation of the seed can be observed due to cell elongation in the integuments. The integuments develop into the seed coat or testa. Halfway through seed development, the embryo growth has been completed and the endosperm has mainly degenerated. By then, the seed is entering the maturation stage in which it prepares for survival and germination. From this stage on, the embryo represents most of the volume of the seed.

The first important aspect of the maturation stage is the accumulation of food reserves that can be hydrolyzed upon germination. In *Arabidopsis*, these reserves

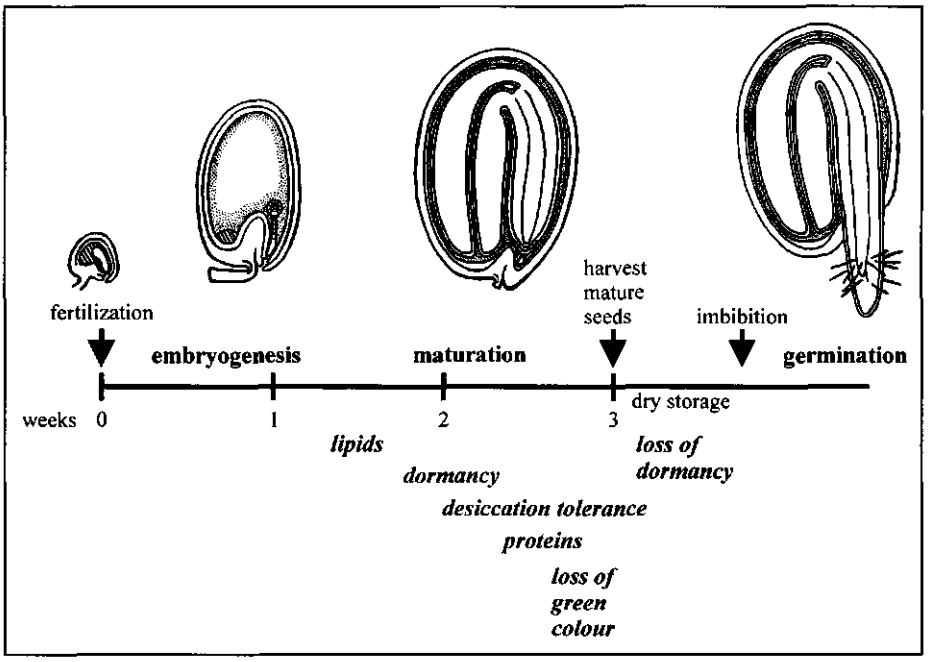

Figure 1.1. Schematic representation of major morphological and physiological changes during seed development and germination in *Arabidopsis*.

are mainly lipids (triacylglycerols with chains of 18-20 C-units) that can make up 30% of the seed (Browse and Somerville, 1994). In addition, seeds accumulate storage proteins. These are located in membrane-bound protein bodies in the embryo. In *Arabidopsis* seeds, 12S (globulin) and 2S (albumin) are abundantly present; these are similar in size and subunit composition to the cruciferin and napin seed storage proteins of *Brassica napus* (Heath et al., 1986). The genes encoding the 12S proteins comprise a gene family of four members (Pang et al., 1988); the 2S proteins are present in 5 isoforms (Krebbers et al., 1988; Van der Klei et al., 1993). In the seeds, starch is also formed, but it can be considered as an intermediate storage compound, since halfway through seed development starch can be detected but at maturity this level has dropped considerably (De Bruin, 1993).

Two other important aspects of seed maturation are the induction of dormancy and desiccation tolerance. Dormancy has been defined as a temporary failure of a viable seed to germinate in conditions that should be favourable for germination with respect to light, humidity and oxygen availability (Simpson, 1990). Dormancy is induced soon after embryogenesis has been completed (Koornneef et al., 1989). Although most ecotypes are dormant to a certain extent,

the degree of dormancy can be highly variable between ecotypes (Koornneef and Karssen, 1994) and is also dependent on environmental conditions (Derkx and Karssen, 1993b). As is shown by the phenotype of various mutants, the degree of dormancy can be determined by the embryo, but also by the testa. For example, the *ttg* and *gl2* mutants combine an altered testa with reduced dormancy (Koornneef, 1981). Desiccation tolerance is established somewhat later in development than dormancy, in L*er* at approximately 14 days after pollination (Koornneef et al., 1989).

Associated with stages in seed maturation, different kinds of particular transcripts (mRNA) can be detected. These comprise the classes of Class I, *MAT*, and *LEA* genes, according to their subsequent timing of expression (Hughes and Galau, 1989; Parcy et al., 1994). The *LEA* (late embryogenesis abundant) genes are supposed to be involved in desiccation tolerance because often these are also expressed in vegetative tissue upon drought stress. Many of the *LEA* genes are inducible by abscisic acid (ABA) and some are involved in other stresses as well, such as cold stress or defence against pathogens (Delseny et al., 1994).

During maturation, a gradual water loss occurs and in the last few days the breakdown of chlorophyll can be observed. At the same time, pigments, derived from flavonolic compounds, accumulate in the testa, giving the seed a brown appearance. A schematic representation of seed development and germination is given in Figure 1.1.

The mutant approach

Mutants are used to study the process that is affected by the mutation. The mutant approach has been very successful in *Arabidopsis* and is leading to great progress in understanding the regulation of physiological processes down to the molecular level. Because of its small size and abundant seed set, *Arabidopsis* is a very appropriate species for mutagenesis. Different kinds of mutagenic sources can be used; chemical mutagens such as EMS or DEB, ionizing radiation like X-rays or γ-irradiation, and insertion of DNA into genes by transposable elements or foreign DNA introduced by transformation. Depending on the kind of mutagen used, several methods can be applied to clone a gene, departing from the mutant phenotype. Since mapping of the genome is very advanced in *Arabidopsis*, there are now many genes isolated via positional cloning, e.g. *ABI3* and *FAD3* (Giraudat et al., 1992; Arondel et al., 1992). Mutagenesis by transposons or T-DNA enables relatively quick cloning of the corresponding gene because the inserted DNA, that

is of known structure, can be used to isolate the genomic DNA surrounding the insertion (Feldmann, 1992; Coupland, 1992; Aarts et al., 1995).

In *Arabidopsis*, seeds are commonly used to mutagenize (M_1 generation). Flowers that originate from the same mutated cell will give a silique with 25% mutant seed (M_2 generation) after self pollination, in the case of a recessive mutation. In the M_2 generation (seed or plant stage) mutants can be identified. For maternally expressed mutations, selection among seeds should take place in the M_3 generation. For example, a testa mutation can only be seen in a seed grown on a mother plant expressing the mutant phenotype because the seed coat originates from maternal tissue. Starting a mutagenesis experiment with mutant seeds and selecting in this mutant background gives the possibility to isolate mutants that enhance or suppress the phenotype of the original mutant. It is important to notice that not every kind of mutant can be found. Mutations may lead to sterility of the plant or give lethality of the embryos or seedlings. Other mutants have no distinguishable phenotype since the gene product is not essential for normal development or the function is taken over by other genes often encoding very similar proteins (Koornneef, 1991). After selection, putative mutants have to be "confirmed" in the next generation. For genetic analysis of mutants the mode of inheritance is studied in the progeny from a cross to the wild-type. In case of seed mutants, the either maternal or embryonic inheritance can be established by studying reciprocal crosses. It is useful to know the map position of a mutant because it is part of its "identification" and this information may be used for the cloning of the corresponding gene. The map position can be determined by linkage analysis with morphological or molecular markers.

Seed development mutants

As described above, seed development can be divided into embryogenesis and maturation. Although these two stages must not be considered too strictly separate, it is a convenient starting point making a classification of seed development mutants. Extensive screens have been designed to isolate embryo-defective mutants from *Arabidopsis* and these have yielded mutants affected in embryogenesis as such, but also mutants that can be considered as seed maturation mutants (Meinke, 1995). Many embryo-lethal mutants are altered in essential "housekeeping" genes; for example, a biotin auxotroph shows disturbed embryogenesis (Schneider et al., 1989). Other mutants can be defective in genes that are responsible for regulation in plant growth and/or development, e.g. mutants disturbed in embryonic pattern

formation can give information about the underlying processes that control plant embryogenesis (Mayer et al., 1991).

From screens for mutants with striking embryonic abnormalities, the class of *leafy cotyledon* mutants has emerged. These mutants exhibit severe defects in seed maturation and produce viviparous embryos with cotyledons that are partially transformed into leaves (indicated by the presence of trichomes) and often accumulate anthocyanin. Initially one locus was described, *lec1* (*leafy cotyledon 1*) (Meinke, 1992), but also the *fus3* (*fusca 3*) mutant that had been isolated in the 1960's on the basis of its dark purple embryos, belongs to the class of *lec* mutants (Bäumlein et al., 1994; Keith et al., 1994; Meinke et al., 1994). In subsequent screens another *lec* mutant (*lec2*) with a less extreme phenotype was found (Meinke et al., 1994). The defects in the *lec* mutants affect many aspects of seed maturation. Only low amounts of proteins and lipids are present, which was also shown in ultrastructural analyses. In mutant embryos, starch granules are present, and few or no protein and lipid bodies. Mutant seeds fail to become dormant and sometimes show precocious germination. In addition, desiccation tolerance is not induced, but when the seeds are allowed to germinate before desiccation they develop into normal plants. This is indicative of the strictly seed-specific nature of these mutations. The *lec* class mutants have been considered as homeotic or as heterochronic mutants. The first model implies that in the absence of normal *LEC* function, mutant cotyledons revert to the primitive, leaflike state (Meinke, 1992). A heterochronic nature of the *lec* mutations implies that the *LEC* genes serve to suppress precocious activation of vegetative development programs which start prematurely in the mutants (Keith et al., 1994). In the *abi3* (*abscisic acid insensitive-3*) mutant as well, several aspects of seed maturation, such as desiccation tolerance and accumulation of storage compounds, are abolished. It is distinguished from the *lec* mutants by a green seed colour and by ABA-insensitivity at germination (Nambara et al., 1992). From analyses of single and double *abi3* and *lec* mutants it has been concluded that normal perception of ABA by *ABI3* is necessary, but not sufficient for seed maturation. A separate, but related group of regulatory factors is encoded by the *LEC* genes (Meinke et al., 1994).

Besides mutants that are disturbed in multiple aspects of seed maturation, mutations that affect specific aspects of seed maturation are also known. Mutants at a number of different loci that affect seed fatty acid composition have been isolated. For many of them no specific effect on seed morphology or physiological characters have been described (James and Dooner, 1990; Lemieux et al., 1990). These are presumably mutations in genes encoding enzymes in the fatty acid

biosynthesis, e.g. the *FAD2* gene encodes a desaturase that is a critical step in the formation of polyunsaturated fatty acids (Okuley et al., 1994). Only the *wri* (*wrinkled*) mutants combine a wrinkled seed phenotype with a severely reduced level of storage lipids, whereas storage protein levels are normal (Focks and Benning, 1996). No mutants are known that have specifically lowered levels of seed storage proteins. Among ecotypes, a large variation in seed size is observed (Krannitz et al., 1991), possibly reflecting genetic differences in aspects of seed development.

Plant hormones

The study of the role of plant hormones using physiological, genetic, and molecular analyses, has revealed the important role of the plant hormone abscisic acid (ABA) in seed maturation. It is a sesquiterpenoid molecule that is derived from an epoxy-carotenoid (Zeevaart and Creelman, 1988). Mutants affected in ABA biosynthesis or ABA response have been isolated in several plant species and all show effects on seed maturation processes. ABA is also involved in stomatal closure and the adaptation to environmental stresses, such as drought adaptation and cold acclimation. The first ABA-deficient (*aba*) mutants in *Arabidopsis* were isolated by means of a selection for germinating seeds among mutagenized non-germinating gibberellin (GA) deficient seeds (Koornneef et al., 1982). Characteristics of ABA-deficient mutants are the absence of seed dormancy and a wilty plant phenotype. These features are also present in ABA response mutants, that are able to germinate on ABA concentrations that are inhibitory for germination of wild-type seeds. Two of these *abi* mutants, *abi1* and *abi2*, are wilty, whereas the phenotype of the *abi3* mutant is strictly confined to seeds (Koornneef et al., 1984). ABA-deficient mutants in other species such as *Nicotiana plumbaginifolia*, tomato, and maize all lack dormancy (Rousselin et al., 1992; Groot and Karssen, 1992; McCarty, 1995). Dormancy is not the only aspect of seed maturation in which ABA is involved. This is indicated by the phenotype of a recombinant that combines ABA-deficiency and ABA-insensitivity (*aba,abi3*). Seeds of this double mutant are green, desiccation intolerant and show reduced levels of storage proteins (Koornneef et al., 1989). Extreme alleles of the *abi3* mutant exhibit this phenotype as well (Nambara, 1992; Ooms et al., 1993).

Of all plant hormones, ABA is the most important plant hormone in the induction of dormancy and other seed maturation processes. For germination however, it is GA which has the most obvious role. GA biosynthesis is required for germination, as has been shown with GA-deficient mutants. This biosynthesis is

light-induced, indicating the involvement of phytochrome in germination (Karssen et al., 1989; Derkx and Karssen, 1993a). ABA and GA have indirect antagonistic effects. In *Arabidopsis* embryos ABA levels peak halfway through seed development and are responsible for the induction of dormancy (Karssen et al., 1983). In ABA-deficient mutant seeds, no dormancy is induced and subsequently the GA requirement for germination is lower (Karssen and Laçka, 1986). The release from dormancy in *Arabidopsis* seeds corresponds with an increase in responsiveness to GA (Karssen and Laçka, 1986; Derkx and Karssen, 1993a).

Ethylene insensitive mutants show a reduced germination (Bleecker et al., 1988), indicating that ethylene may be involved in germination. Ethylene can induce germination in GA deficient *Arabidopsis* seeds (Karssen et al., 1989). Germination is accompanied by ethylene production in many plant species; for some species this is shown to be essential for germination or the breakage of dormancy, but for others there is no requirement for endogenous ethylene during germination (Lalonde and Saini, 1992).

A phenomenon that is not well understood is the cross resistance of some hormone mutants. An ABA-deficient mutant of *N. plumbaginifolia* shows cross resistance at the seed germination level to cytokinin and auxin, but this does not apply to *Arabidopsis* (Rousselin et al., 1992). Auxin resistant mutants of *Arabidopsis* show cross resistance to ABA and/or ethylene with respect to root growth (Pickett et al., 1990; Wilson et al., 1990). This may mean that some links exist between the signal transduction pathways of several plant hormones, including ABA. Hypotheses in this respect can be that a "receptor" is stimulated by one and inhibited by another hormone or that one hormone may act by altering the level or sensitivity to a second hormone (Klee and Estelle, 1991).

Aim and outline of this study

In order to extend the genetic analysis of seed development and in particular seed maturation in *Arabidopsis*, mutant screens have been designed to obtain novel mutants affecting this process. Mutagenesis was performed with EMS, γ-irradiation, and transposons, on wild-type and *abi3* seeds. These screens have resulted in the selection of seeds that germinate in the presence of ABA, GA biosynthesis inhibitors or high NaCl concentrations. Furthermore, screens have resulted in the selection of green or anthocyanin-accumulating seeds, seeds with a reduced or enhanced dormancy, or extreme seed sizes. Finally, mutants were selected in which the expression of reporter genes driven by a seed specific promoter is altered. The mutants have been characterized genetically and physiologically.

One aspect of seed maturation that is affected in all mutants analyzed, is seed dormancy. As mentioned before, the degree of dormancy can be determined by the embryo, but also by the testa. The latter situation applies to a mutant that has an abnormal integument development, leading to aberrantly shaped seeds with a testa consisting of fewer cell layers than wild-type seeds. The genetic and morphological analysis of this mutant is described in Chapter 2. A number of new alleles of the *abi3* mutant has been found. Some of these exhibit a severely disturbed seed maturation and were, therefore, useful to investigate what factors are important for the induction of desiccation tolerance (Chapter 3). The *abi3* mutants were also included, together with mutants from the *lec* class, in a comparison of seed maturation mutants with respect to storage compound accumulation and dormancy and germination behaviour (Chapter 4). Chapter 5 describes the isolation and physiological characterization of additional ABA biosynthetic mutants. A direct screen for reduced dormancy yielded mutants at two separate loci that control dormancy in a hormone-independent way (Chapter 6).

CHAPTER 2

A seed shape mutant of *Arabidopsis*, which is affected in integument development

Karen M. Léon-Kloosterziel
Christian J. Keijzer
Maarten Koornneef

Summary

A seed shape mutant of Arabidopsis was isolated from an ethyl methanesulfonate-treated population. Genetic analysis revealed that the heart shape phenotype was maternally inherited, showing that this is a testa mutant. This indicates the importance of the testa for the determination of the seed shape. This recessive aberrant testa shape (ats) gene was located at position 59.0 of chromosome 5. A comparison was made between ovules and developing and mature seeds of the wild-type and of the mutant using light and scanning electron microscopy. It was shown that the mutant seed shape is determined during the first few days after fertilization, when the embryo occupies only a very small part of the seed. The integuments of ats ovules consisted of only three rather than five cell layers. In double mutants, the effect of ats was additive to other testa mutations, such as transparent testa, glabra (ttg), glabrous2 (gl2), and apetala2 (ap2). The ats mutation resulted in a reduced dormancy, which was maternally inherited. This effect of a testa mutation on germination was also seen in ttg seeds, in which the outer layer of the testa was disturbed. This indicated the importance of the testa as a factor in determining dormancy in Arabidopsis.

Introduction

Seeds can be divided into parts of a genetically different origin: an embryo, the endosperm, and the seed coat or testa. The zygote, from which the embryo develops, combines the genotypes of the haploid male and female gametes. The embryo development in *Arabidopsis* is well documented: it follows the classic *Capsella* variation of the Onagrad type (Misra, 1962; Mansfield and Briarty, 1991). The endosperm is a result of a triple fusion. The two haploid polar nuclei of the central cell fuse, and, subsequently, this nucleus is fertilized by a haploid sperm nucleus, resulting in a triploid endosperm nucleus (Mansfield et al., 1991). This nucleus starts to divide, giving rise to a coenocytic endosperm. Not until the embryo is in the late globular stage does the endosperm begin to cellularize (Mansfield and Briarty, 1990). In mature seeds, the aleurone layer represents the remainder of the endosperm (Müller, 1963). After fertilization, during embryo development and subsequent seed maturation, the integuments undergo morphological changes and become the testa, which accordingly has the maternal genotype. Recently, ovule development in *Arabidopsis* has been described extensively (Robinson-Beers et al., 1992; Reiser and Fischer, 1993). The initiation of the integuments in *Capsella bursa-pastoris* has been described by Roth (1957). Bouman (1975) reported on testa development in some Cruciferae.

In *Arabidopsis*, various mutations in genes controlling different aspects of ovule and seed development have been described. Embryonic mutants can be defective in essential housekeeping genes or in genes involved in the regulation of embryo development (Meinke, 1991). Mutants with specific defects in pattern formation during embryogenesis are valuable tools in the elucidation of early developmental processes (Mayer et al., 1991). Ovule mutations, such as *short integuments* (*sin1*), *bell* (*bel1*) (Robinson-Beers et al., 1992), *ovule mutant-2* (*ovm2*), and *ovule mutant-3* (*ovm3*) (Reiser and Fischer, 1993), result in female sterility. Another category of seed mutants that are maternally inherited but expressed at later stages of seed development are testa mutants. *Transparent testa* (*tt*) mutants lack the brown pigment in the seed coat and often, but not always, lack anthocyanin in their vegetative tissues as well (Koornneef, 1990). In addition to these characteristics, the *transparent testa, glabra* (*ttg*) mutant is impaired in its ability to form trichomes and has a disturbed seed surface, which are features exhibited also by the *glabrous-2* (*gl2*) mutant (Koornneef, 1981). It has been suggested that the *TTG* gene is a homologue of the *R* locus in maize, which is a

transcription factor that activates promoters of biosynthetic genes in the anthocyanin pathway (Lloyd et al., 1992).

Here, we describe the isolation and the genetic, morphological, and physiological characterization of an *Arabidopsis* testa mutant with seeds that are heart shaped rather than having the normal oblong shape.

Results

Genetic characterization

A mutant with heart-shaped seeds was isolated from an M_3 population. Genetic analysis revealed that this mutation was maternally inherited: if a mutant plant was pollinated with wild-type pollen, the F_1 seeds had the mutant phenotype. If a wild-type plant was pollinated with mutant pollen, the F_1 seeds had the normal, elongated seed shape. All F_2 seeds had this wild-type phenotype as well. In an F_2 generation, 186 plants with wild-type seeds and 47 plants with mutant seeds were found. These data fit a 3:1 ($\chi^2 = 2.78$; $P > 0.05$) segregation ratio, indicating that this is a single recessive mutation. This progeny analysis showed that the maternal heredity is not due to cytoplasmatic heredity or maternal imprinting. Therefore, this mutation apparently affects the testa, and the locus was designated *aberrant testa shape* (*ats*). Linkage analysis using F_2 and F_3 data from crosses with the chromosome 5 markers *ttg*, *yellow inflorescence* (*yi*) and *abscisic acid-deficient* (*aba*) revealed significant linkage between *ats* and *ttg* and between *ats* and *yi* (Fig. 2.1), locating the *ats* mutation at position 59.0 on chromosome 5.

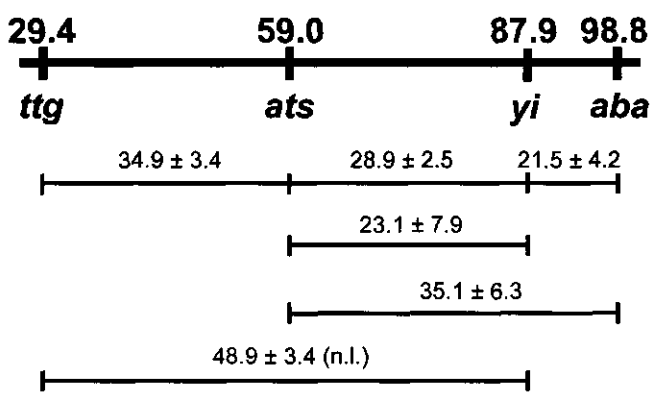

Figure 2.1. The location of *ats* on the chromosome 5 map. The map locations on chromosome 5 of *ttg*, *ats*, *yi*, and *aba* (in centiMorgan) are indicated on the top bar. The estimates of recombination percentages with standard deviations between *ats* and the morphological markers *ttg*, *yi*, and *aba* are shown below the top bar. n.l., no linkage.

Morphology of ovules and seeds

Scanning electron microscopy (SEM) and light microscopy were used to compare the development of ovules and seeds of the wild-type with that of the *ats* mutant. Figure 2.2, A and C, illustrates that in wild-type ovule primordia, two distinct rims of cells appear, developing into the inner and outer integument. Figure 2.2, B and D, shows that the development of the integuments of *ats* ovules is irregular. During the development of *ats* ovules, no clear distinction between the developing inner and outer integument can be seen. However, mutant and wild-type ovules have the same overall shape (Fig. 2.2, E and F).

Both in wild-type and in mutant ovules, rapid expansion of the endosperm immediately after fertilization was observed (Fig. 2.2, G and H). The expansion of the testa was merely caused by cell elongation and not by cell division, because the number of cells of the integuments did not increase. During this process, occurring during the first 4 days after anthesis, the wild-type seeds became oval, but *ats* seeds maintained their roundish shape. Figure 2.3 shows that seeds at 4 days after anthesis have reached the shape of a mature seed and have nearly reached their ultimate size. At this stage, the embryo was in the globular stage and occupied only a very small part of the seed. No differences in the shape of the embryos between the wild-type and the *ats* mutant were observed during the first few days after anthesis.

In Figure 2.4, cleared ovules and SEM sections of ovules at 3 days after anthesis show the cell layers of the integuments. When the embryo is in the globular stage, the endosperm cellularization has not yet begun, so all visible layers are testa layers (Mansfield and Briarty, 1990). In wild-type ovules, the outer two cell layers represent the outer integument, which has overgrown the shorter inner integument, thereby forming the micropyle. The inner three cell layers represent the inner integument. The innermost cell layer of the inner integument ultimately becomes the pigmented layer of the mature seed (Bouman, 1975). In contrast to the wild-type, in which the testa consisted of five cell layers at the apical end (Fig. 2.4B), the testa of *ats* seeds consisted of only three cell layers at this position (Fig. 2.4D). Apparently, two cell layers are absent in mutant seeds. In cleared *ats* ovules, the structure of the innermost cell layer was very similar to this corresponding layer in wild-type ovules and because *ats* seeds were normally pigmented, this indicated that this layer was unaffected in *ats* ovules.

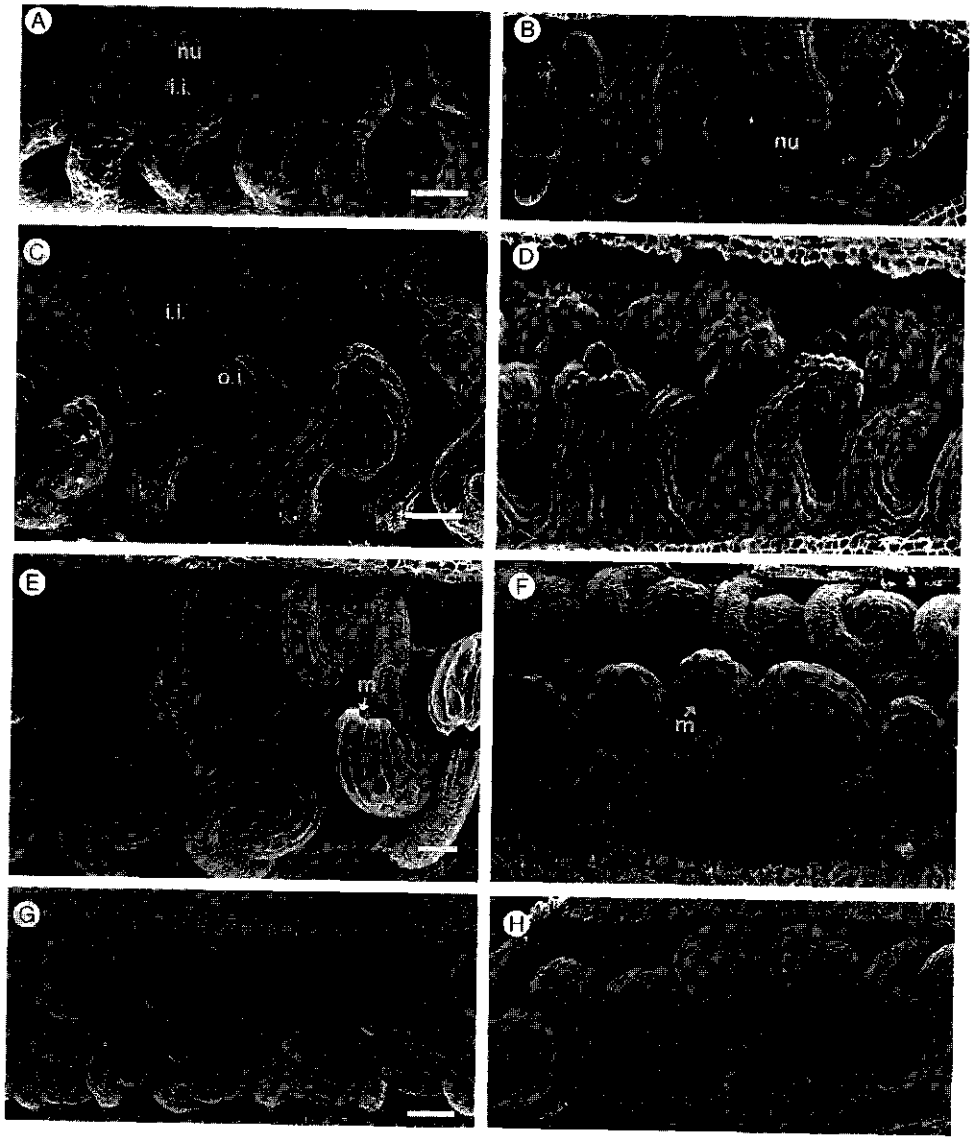

Figure 2.2. Scanning electron micrographs of developing wild-type and mutant ovules. Developmental stages refer to stages as described by Smyth et al. (1990). A, C, E, G, wild-type ovules; B, D, F, H, *ats* mutant ovules.
A and B, stage 11.i.i., inner integument; nu, nucellus. Bars = 25 µm
C and D, stage 12 early. i.i., inner integument; o.i., outer integument. Bars = 25 µm.
E and F, stage 13: ovules at the day of anthesis. m, micropyle. Bars = 25 µm.
G and H, ovules one day after anthesis. Bars = 50 µm.

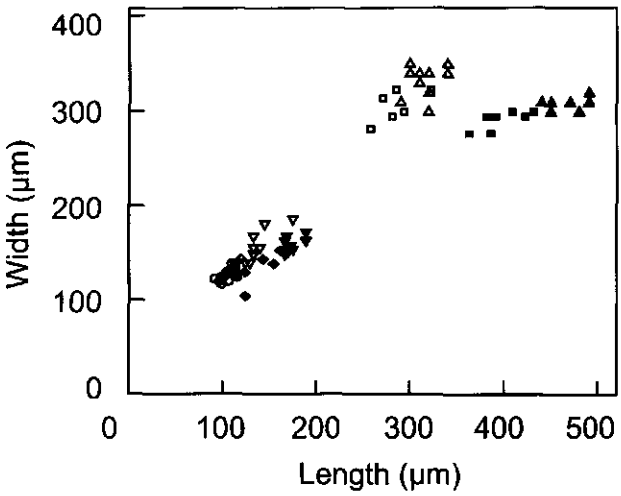

Figure 2.3. Length-width relation of ovules and seeds. Lengths plotted against widths of ovules at the day of anthesis (○,●), at 1 day (◊,◆), 2 days (▽,▼), and 4 days (□,■) after anthesis, and of mature seeds (△,▲). The closed symbols show the wild-type and the open symbols show the *ats* mutant.

The surface of *Arabidopsis* wild-type seeds consists of polygonal structures with a central elevation, the columella (Fig. 2.5, A and B). Wild-type seeds excrete a layer of mucilage upon contact with water; this layer can be visualized by staining the mucilage with ruthenium red. Figure 2.5, C and D, shows that polygonal structures can be easily recognized on mature *ats* seeds. However, they were irregularly shaped and larger than those of wild-type seeds. Because these structures represent cells of the outer layer, this implies that this layer is present but that these cells are larger in *ats* than in wild-type seeds, as well as being reduced in number. *ats* seeds produced very little mucilage.

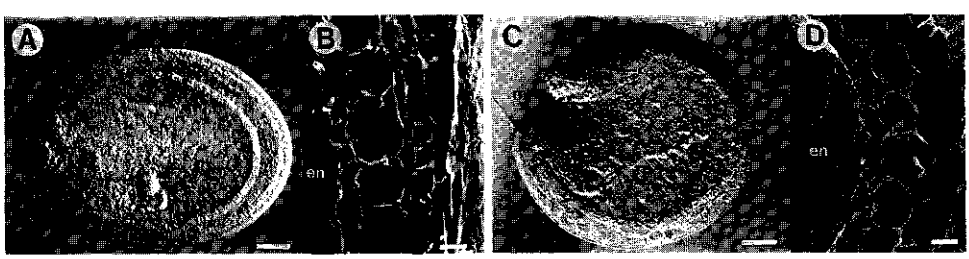

Figure 2.4. Light micrographs and SEM photographs of ovules at 3 days after anthesis. A and B, wild-type ovules. C and D, ovules from an *ats* plant.
Bars in A and C = 50 µm; bars in B and D = 10 µm. e, embryo; en, endosperm; m, micropyle.

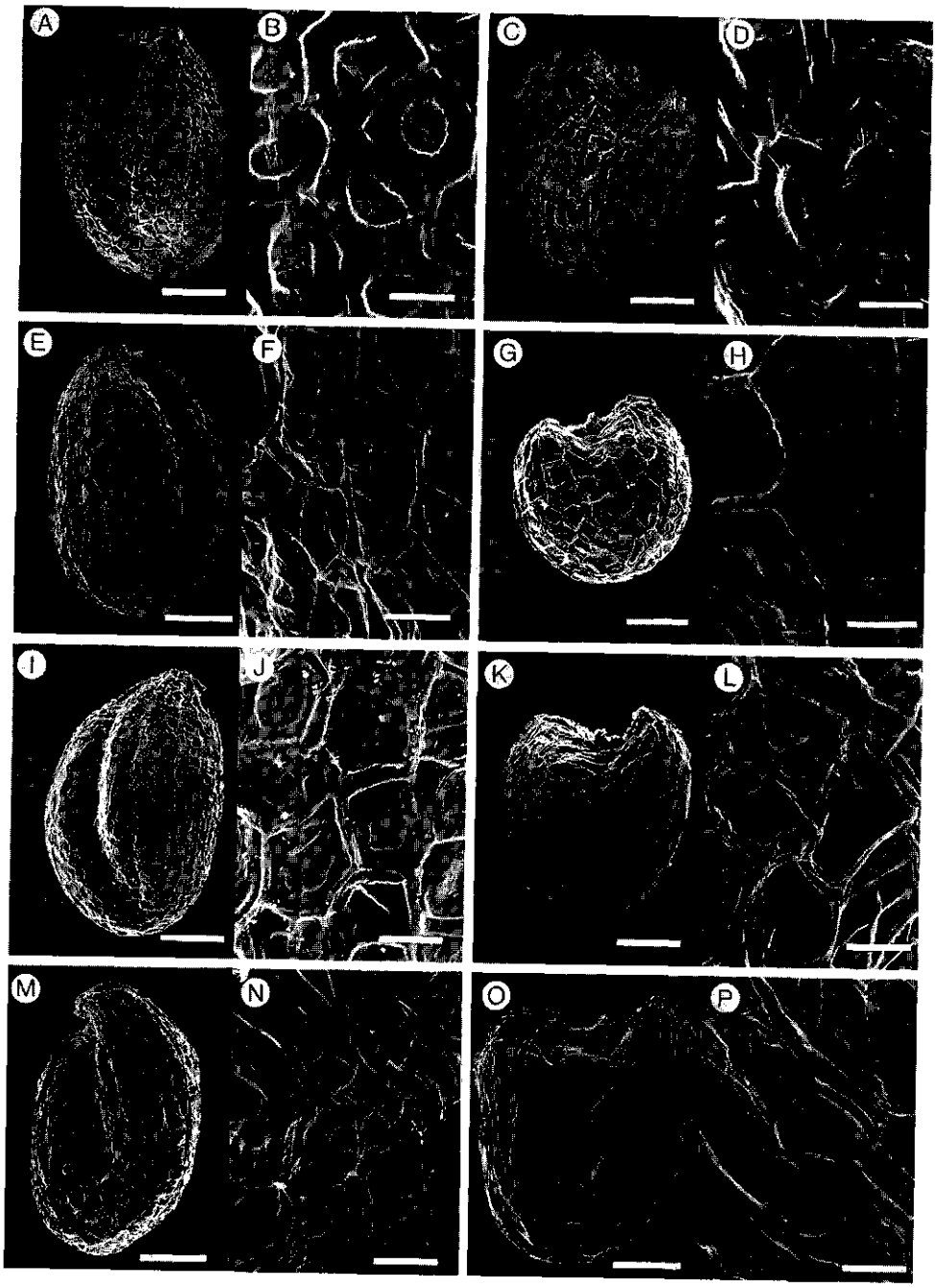

Figure 2.5. Scanning electron micrographs of mature seeds. A and B, wild-type seeds. C and D, *ats* seeds. E and F, *gl2* seeds. G and H, *ats,gl2* double mutant seeds. I and J, *ttg* seeds. K and L, *ats,ttg* double mutant seeds. M and N, *ap2-1* seeds. O and P, *ats,ap2-1* double mutant seeds. Bars in A, C, E, G, I, K, M, and O = 100 µm. Bars in B, D, F, H, J, L, N, and P = 20 µm.

Interaction with other testa mutants

The mutations *gl2* (Fig. 2.5, E and F) and *ttg* (Fig. 2.5, I and J) also result in an aberrant seed coat. In *gl2* and *ttg* seeds, the outer layer is affected because the columellas and the mucilage are absent (Koornneef, 1981). In the *ats,gl2* and *ats,ttg* double mutants, an additive phenotype could be observed (Fig. 2.5, G, H, K, and L), indicating that the *ats* mutation affects the testa in a different process than do *ttg* and *gl2*. The *apetala-2* (*ap2-1*) mutation has a pleiotropic effect on the seeds: *ap2-1* seed shape could vary from the normal oblong shape to a variety of aberrant shapes (Fig. 2.5M). Therefore, some *ap2-1* seeds resembled *ats* seeds. On most *ap2-1* seeds, the surface pattern was not normal (Fig. 2.5N). The polygonal structures were only present on parts of the seed surface and lacked the columella. Seeds of the *ats,ap2-1* double mutant also showed an additive phenotype, because the seed malformation was more extreme (Fig. 2.5, O and P).

Seed germination characteristics and fertility

Viable Landsberg *erecta* seeds, which are freshly harvested, do not germinate under conditions of sufficient oxygen, water, and light supply: they are dormant. Mutants with a transparent seed coat (*ttg*) are known to have a reduced seed dormancy (Koornneef, 1981). Because dormancy is relieved during dry storage of seeds, the germination percentage and germination speed of *ats*, *ttg*, and *ats,ttg* double mutant seeds was determined after seeds were stored for different periods. This allowed us to distinguish the germination characteristics of those genotypes; the results are given in Figure 2.6. Wild-type and *aba* seeds served as dormant and non-dormant controls. Seeds of the *aba* mutant lack dormancy because of the reduced level of abscisic acid (Koornneef et al., 1982).

Three days after harvest, wild-type seeds were fully dormant (0% germination after 7 days of incubation), whereas the non-dormant *aba* seeds germinated within 3 days (Fig. 2.6A). Figure 2.6A shows the severely reduced seed dormancy of *ttg* seeds; *ats* seeds had a slightly reduced dormancy. The germination behaviour of *ats,ttg* double mutants is indicative of the additive effect of both mutations at the physiological level. Release from dormancy was faster in *ats* seeds than in wild-type seeds. After 10 days of storage, 80% of the *ats* seeds had germinated after 7 days of incubation, whereas none of the wild-type seeds had germinated (Fig. 2.6B). Seventeen days after harvest of the seeds, all mutants germinated within 3 days, while a large part of the wild-type seeds had also been released from dormancy (Fig. 2.6C). Thus, *ats* has a reduced seed dormancy. Figure 2.7 shows that the reduced seed dormancy was maternally inherited, indicating that this is determined by characteristics of the testa and not by characteristics of the embryo.

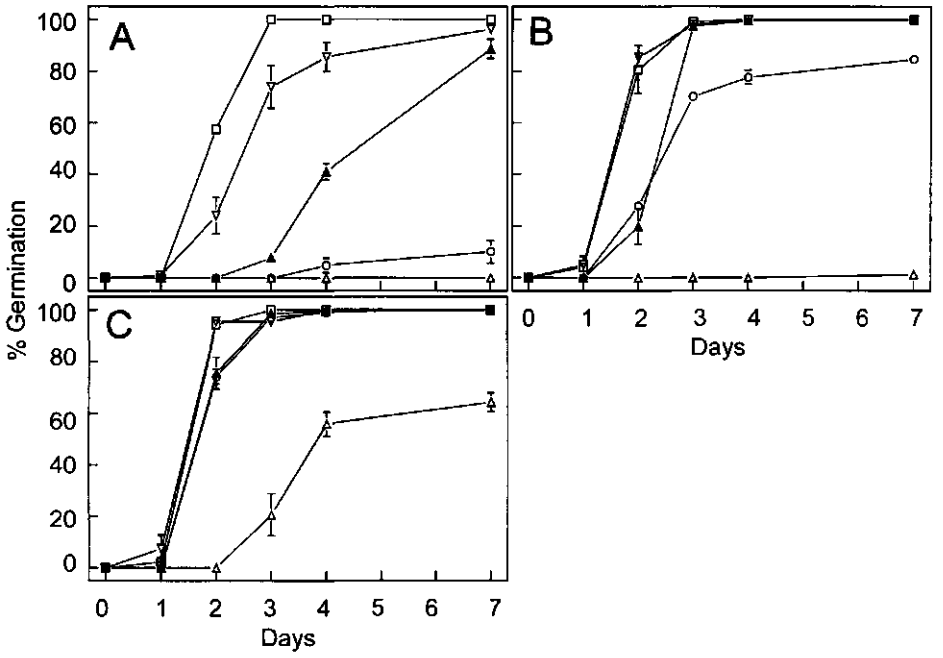

Figure 2.6. Comparison of germination and dormancy of wild-type and testa mutants. The time course of germination of wild-type (△), *ats* (○), *ttg* (▲), *ats,ttg* (▽) and *aba* (□) seeds was determined after different storage periods. A, storage period of 3 days. B, storage period of 10 days. C, storage period of 17 days.

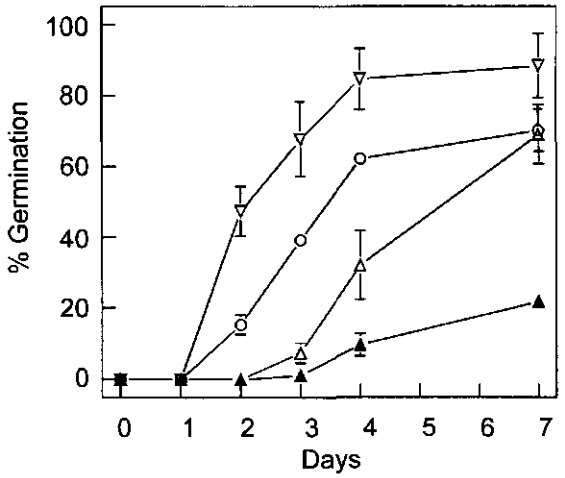

Figure 2.7. Time course of seed germination. Seeds were stored for 17 days. Germination was determined for wild-type (△), *ats* (○), F_1 wild-type x *ats* (▲), and F_1 *ats* x wild-type (▽) seeds.

In addition to the seed characteristics, no pleiotropic effects on the *ats* plants was observed, except that *ats* plants had shorter siliques than did wild-type plants. As shown in Table 2.1, the number of ovules in *ats* siliques was not different from that of wild-type siliques, but the number of mature seeds was lower in *ats* siliques. Since there is a correlation between seed number and silique length (Barendse et al., 1986), the shorter siliques of *ats* plants were the result of a lower seed number per silique. Reciprocal crosses did not indicate that male fertility of the *ats* mutant was affected (data not shown).

Discussion

In this study, we describe the isolation and characterization of an *Arabidopsis* mutant that has a maternally inherited aberrant seed shape (Fig. 2.5C). In this *ats* mutant, only the seed shape is affected; this is in contrast with *ap2-1* for example, in which the seeds have a deviant shape (Fig. 2.5M), and the development of flowers is also affected (Bowman et al., 1989).

The maternal inheritance of *ats* showed that the shape of *ats* seeds is determined by the testa and not by the embryo, notwithstanding the fact that in mature *Arabidopsis* seeds the embryo fills up the seed completely and that the seed coat seems to follow the contours of the embryo. The testa in *Arabidopsis* develops from a two-layered outer integument and a three-layered inner integument (Fig. 2.4B). This showed that not only is the embryogenesis of *Arabidopsis* similar to the embryogenesis of *C. bursa-pastoris* (Mansfield and Briarty, 1991), but that the testa morphology of *Arabidopsis* is also the same as has been observed in *C. bursa-pastoris* (Bouman, 1975).

Light and scanning electron microscopy revealed that *ats* produces ovules in which the integuments do not develop properly. In wild-type ovule primordia, two distinct rings of cells appear, developing into the inner and outer integument (Fig. 2.2A; Robinson-Beers et al., 1992; Reiser and Fischer, 1993). This is also similar to the situation in *C. bursa-pastoris*: Roth (1957) reported that first the inner and subsequently the outer integument develops from two different rings of dermal cells. This clear distinction between the developing integuments is absent in *ats* ovules (Fig. 2.2, B and D), and results in ovules in which the embryo sac is surrounded by three rather than five cell layers (Fig. 2.4). A possible explanation for these missing layers is that in *ats* ovule primordia, only one ring of dermal cells develops or that the two integument primordia are fused and give rise to one integument, which has

Table 2.1. Silique length, number of ovules, and number of mature seeds in siliques of wild-type and *ats* plants.

	Average silique length (mm)	Average number of ovules per silique half	Average number of mature seeds per silique half
wild-type[a]	12.6 ± 0.7	32.2 ± 3.1	30.9 ± 3.2
ats[a]	10.4 ± 0.7	32.2 ± 2.5	26.2 ± 3.4

Means are given ± S.D.
[a] n=23

characteristics of both the inner and outer integument. The aberrant shape of *ats* seeds is established in the first few days following anthesis, when a rapid expansion of the ovules occurs. As a consequence of deviant cell divisions, leading to missing layers, the integuments also may not exhibit proper cell expansion, which results in the aberrant shape.

The *ats* mutant is a valuable addition to the other *Arabidopsis* ovule mutants *Arabidopsis*, *bell*, *sin1* (Robinson-Beers et al., 1992), *ovm2*, and *ovm3* (Reiser and Fischer, 1993). These mutants are impaired in the formation of normal integuments and embryo sacs. It is hypothesized that the inner integument is missing from the ovules of the *bell* mutant. Hybrids of the *ats* and *bell* mutants were fertile and had seeds with the wild-type phenotype, indicating that both mutants were not allelic. This indicates that *ATS* may be a new locus that is required only for integument initiation and not for megagametogenesis, because *ats* is not female sterile. Segregation of F_2 plants bearing *ats* seeds fitted a 3:1 ratio, indicating that the viability of *ats* embryos was not affected. The additive effect of *ats* with the other testa mutations *ttg*, *gl2*, and *ap2-1* indicated that the formation of the integuments is a complex process requiring several genes to determine aspects of cell division planes and cell differentiation.

The germination behaviour of *ats* and *ttg* showed that the degree of dormancy of a seed is determined not only by characteristics of the embryo but also by characteristics of the testa. At germination, the radicle has to penetrate the seed coat, and apparently the structure determining the solidity of the testa is a factor that influences germination. The role of the mucilage must also be considered. Germination of seeds of *Blepharis persica* can be stimulated by removing the mucilage or the seed coat and by increasing the percentage of oxygen to which they are exposed (Witztum et al., 1969). Pricking the seeds or removing the testa also

promotes germination of *Arabidopsis* seeds (Kugler, 1951). A large increase in oxygen uptake rate occurs at the start of germination of seeds of *Sisymbrium officinale*, a species closely related to *Arabidopsis* (Derkx et al., 1993). Perhaps the absence of mucilage on *ats* and *ttg* seeds allows more oxygen to diffuse into the seed, and this higher oxygen level might be able to release it from dormancy. To explain the additive effect of *ttg* and *ats* on germination, one has to assume that the structure of the testa influences germination.

The *ats* mutant will be useful in the study of integument initiation because specific layers of the integuments and, thereby, the testa are affected. Detailed light microscopy of sections of ovules at the stage of integument initiation will more clearly show how the integuments develop in the *ats* mutant. By using testa mutants the function of the testa for dormancy and germination can be explored.

Materials and methods

Mutant isolation and genetic analyses

Mutant lines were generated in *Arabidopsis thaliana* ecotype Landsberg *erecta* (L*er*) seeds carrying the *transparent testa, glabra* (*ttg*) mutation by applying 15 mM ethyl methanesulfonate for 24 hr. In a selection program designed to isolate seed dormancy mutants the seeds from one putative mutant that was selected in the M_2 generation appeared to be heart shaped. This mutant, which had a limited seed set, was backcrossed twice with wild-type L*er*. Fertile plants with heart-shaped brown seeds could be obtained and were used for the microscopic and physiological analysis.

For genetic analysis, the *aberrant testa shape* (*ats*) mutant was reciprocally crossed with the wild-type. For mapping, a cross was made between *ats* and a line homozygous recessive for the markers *ttg* and *yellow inflorescence* (*yi*). Subsequently, a cross was made between an *ats,yi* recombinant and a line homozygous recessive for the markers *ttg* and *abscisic acid-deficient* (*aba*). F_2 and F_3 populations derived from these crosses were scored for seed shape, together with the marker phenotypes. Recombination percentages were estimated using the RECF2 program (Koornneef and Stam, 1992). The map locations were determined with the JOINMAP program (Stam, 1993) by using the data obtained with the present analyses in combination with the data set for classic genetic markers used by Hauge et al. (1993). Correction for double cross-overs was done with the Kosambi mapping function. Double mutants were constructed by crossing the mutant with

lines carrying the mutations *ttg*, *glabrous2* (*gl2*), and *apetala2* (*ap2-1*), respectively. F_2 plants with heart-shaped seeds, but otherwise a wild-type phenotype, were selected. Double mutants were selected from the selfed progeny of these F_2 plants that segregated for *ttg*, *gl2*, or *ap2-1*, respectively.

Microscopy

For scanning electron microscopy, siliques and flowers were immersed in 2% glutaraldehyde for 16-20 hr at room temperature and subsequently dehydrated in a graded series of ethanol. Critical point drying was carried out in liquid carbon dioxide. The siliques and flowers were mounted on stubs, dissected using a special microtome (Keijzer, 1993), and sputter coated with paladium-gold. Specimens were examined with a scanning microscope (model JSM 5200, Jeol, Tokyo, Japan). For light microscopy, ovules were removed from the pistil or the silique and immersed in a droplet of clearing solution (72% (w/v) chloral hydrate, 17% (w/v) water, 11% (w/v) glycerol) on a slide and covered with a coverslip. The ovules were examined with a microscope (Nikon) equipped with Nomarski optics.

Germination assay

For germination assays, mature seeds were harvested from dehydrated siliques. After storage for 3, 10, or 17 d at room temperature, the seeds were sown on water-saturated filter paper (No. 595, Schleicher & Schuell) in petri dishes and incubated at 21°C under continuous white light (Philips TL57 and incandescent bulbs). The seeds were scored for germination every day during a 7-day period.

Culture conditions

To grow plants, seeds were sown in petri dishes on water-saturated filter paper and incubated in a growth chamber at 25°C. After 2 d of incubation, germinated seeds were transferred into soil and cultivated in an air-conditioned greenhouse (18-23°C) with additional light during the winter (Philips HPI-T/400W; 16-hr photoperiod).

Acknowledgements

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CHAPTER 3

Acquisition of desiccation tolerance and longevity in seeds of *Arabidopsis thaliana*. A comparative study using aba-insensitive *abi3* mutants

Jaap J.J. Ooms
Karen M. Léon-Kloosterziel
Dorothea Bartels
Maarten Koornneef
Cees M. Karssen

Summary

Two new abscisic acid (ABA)-insensitive mutants of Arabidopsis thaliana affected in the abi3 locus are described. These new mutants are severely ABA-insensitive. Like the earlier described abi3-1 and the ABA-deficient and -insensitive double mutant, aba1-1,abi3-1, these new mutants vary in the extent of ABA-correlated physiological responses. Mutant seeds fail to degrade chlorophyll during maturation and show no dormancy, and desiccation tolerance and longevity are poorly developed. Carbohydrate accumulation as well as synthesis of LEA or RAB proteins are often suggested to be essential for acquisition of desiccation tolerance. In this work two points are demonstrated. (a) Accumulation of carbohydrates as such does not correlate with acquisition of desiccation tolerance or longevity. It is suggested that a low ratio of mono- to oligosaccharides rather than the absolute amount of carbohydrates controls seed longevity or stability to desiccation tolerance. (b) Synthesis of a few assorted proteins, which is responsive to ABA in the later part of seed maturation, is not correlated with desiccation tolerance or longevity.

Introduction

ABA plays an important role in the development and maturation of seeds (Black, 1991). This hormone is essential for the induction of seed dormancy, as shown in experiments with ABA-deficient mutants (Karssen et al., 1983), and it is associated with the acquisition of desiccation tolerance during seed development (Koornneef et al., 1989). The ABA-deficient and -insensitive mutants of *Arabidopsis thaliana* enable the elucidation of crucial factors involved in desiccation tolerance. Three different loci for ABA-insensitivity, designated *abi1*, *abi2*, and *abi3*, have been described (Koornneef et al., 1984). The *abi1* and *abi2* mutants suffer from disturbed water relations in the vegetative stage (i.e. wilting under water stress), whereas the *abi3* mutation specifically affects seed development (Koornneef et al., 1984). Seeds have reduced amounts of storage proteins and eicosenoic acid, the main fatty acid component of storage lipids (Finkelstein and Somerville, 1990). Seeds of ABA-deficient as well as ABA-insensitive mutants acquire desiccation tolerance, which may be due to leakiness of the mutations. However, the construction of the ABA-deficient and -insensitive double mutant *aba1-1,abi3-1* resulted in plants that produce viable but desiccation-intolerant seeds. This desiccation-sensitive genotype allows a detailed analysis of desiccation tolerance in seeds (Koornneef et al., 1989; Meurs et al., 1992).

In several reports on desiccation tolerance, the main focus has been on carbohydrates and proteins (Kermode, 1990; Leopold, 1990; Skriver and Mundy, 1990). Carbohydrates may play an essential role in the acquisition of desiccation tolerance (Crowe et al., 1984; Leopold, 1990). One of the suggested functions of carbohydrates is membrane protection upon dehydration. Withdrawal of water molecules from the phospholipids can lead to membrane phase transitions at physiological temperatures (Crowe et al., 1989; Hoekstra et al., 1989). When water is available, these phase transitions coincide with membrane leakage and cell death. Hoekstra et al., (1991) have shown that carbohydrates can suppress the temperature of phospholipid phase transitions and prevent leakage of cellular solutes. In addition to the protection of phospholipids, carbohydrates are involved in the stabilization of proteins and retention of enzymatic activity during dehydration (Carpenter and Crowe, 1988).

A second mechanism in which sugars are probably involved, is in the formation of a glass during dehydration (Burke, 1986). A glass is a liquid of high viscosity, such that it stops all chemical reactions requiring molecular diffusion and might function in conserving tissue structures during dehydration. Comparing desiccation-tolerant and -intolerant tissues, Bruni and Leopold (1991) found that all tolerant tissues had formed a glassy state, whereas the intolerant tissues had not. *In*

vitro studies have shown that the relative amounts of different sugars can influence the stability of the glassy state at physiological temperatures (Koster, 1991).

Besides carbohydrates, the synthesis of specific proteins might also be involved in the acquisition of desiccation tolerance. From studies of the *aba1-1,abi3-1* double mutant it is known that accumulation of storage proteins is inhibited (Koornneef et al., 1989; Meurs et al., 1992). Instead of accumulating maturation-related proteins, *aba1-1,abi3-1* seeds synthesize germination-related proteins in the later stages of development (Meurs et al., 1992). The absence of maturation-related proteins in *aba1-1,abi3-1* seeds agrees with the general view that ABA and/or dehydration stimulate the synthesis of proteins that are involved in the protection from desiccation damage (Skriver and Mundy, 1990; Black, 1991).

In this study, we introduce two new *abi3* mutant alleles that allow normal plant growth, but keep seeds green until maturity. The mutants are characterized by a reduced desiccation tolerance and/or longevity. The seed phenotypes of these new ABA-insensitive mutants strongly resemble the recently described *abi3-3* mutant seeds (Nambara et al., 1992) and the *aba1-1,abi3-1* double mutant seeds (Koornneef et al., 1989). The *abi3* gene has recently been cloned (Giraudat et al., 1992) and encodes a protein with distinct regions of homology to the maize *vp1* product (McCarty et al., 1991) which is suggested to be a transcriptional activator and may potentiate ABA responses in the maize embryo. We studied the effect of ABA-insensitivity on dormancy development, acquisition of desiccation tolerance and longevity in seeds with three different *abi3* alleles, and in seeds of the *aba1-1,abi3-1* double mutant and wild-type. The use of mutants that are gradually different in ABA-insensitivity facilitates the elucidation of the role of carbohydrates and proteins in the acquisition of desiccation tolerance and longevity.

Results

Genetic analysis of the SM1 and 10286 mutant

To test allelism, crosses were made between 10286 and SM1 and between 10286 and *abi3-1*. The F_1 seeds from these crosses germinated within 2 to 3 d and, in addition, were able to germinate in the presence of 10 µM ABA. Lack of dormancy and this insensitivity to ABA demonstrate allelism. Linkage analysis using F_2 data from crosses between SM1 and marker lines revealed linkage between the SM1 mutation and *hy2* and *gl1* with estimates of recombination percentages of 31.3 ± 2.3 and 8.6 ± 1.1 centiMorgan, respectively. These linkage data are in accordance with the previously published map position of *abi3* (Koornneef and Hanhart, 1984). These data together indicate that the mutant lines SM1 and 10286 have mutations in

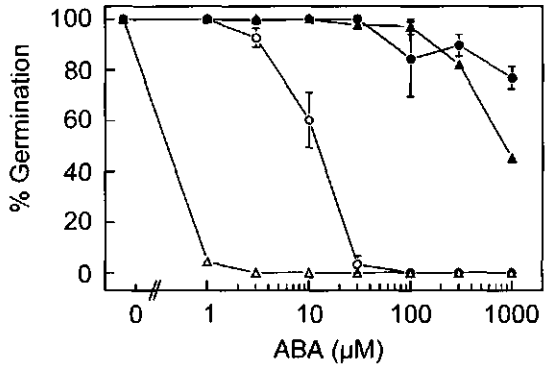

Figure 3.1. Germination capacity in the presence of ABA. Freshly harvested seeds from mature siliques of wild-type (△) and the mutants *abi3-1* (○), *abi3-4* (▲), and *abi3-5* (●), were tested for their ability to germinate in different ABA concentrations. Data are averages ± SD of triplicate determinations.

the *ABI3* locus. We designate these new alleles *abi3-4* (SM1) and *abi3-5* (10286).

Germination tests

For a preliminary physiological characterization of the various *abi3* mutants in comparison with wild-type and the *aba1-1,abi3-1* double mutant seeds, we tested germination behaviour under different conditions and at different stages of development. Figure 3.1 shows the sensitivity of mature seeds to the ABA-induced inhibition of germination. It is clear that germination of wild-type seeds is fully inhibited at 10 μM ABA, whereas similar inhibition of *abi3-1* seed germination occurs at 100 μM ABA. In contrast, germination of seeds from both new *abi3* mutations (*abi3-4* and *abi3-5*) could be only slightly inhibited by 1 mM ABA. This strong insensitivity to ABA of the *abi3-4* and *abi3-5* mutant seeds correlates with the more severe phenotype of the *abi3-4* and *abi3-5* alleles as compared with the *abi3-1*. The alleles strongly resemble the green seeds of the ABA-insensitive and -deficient double mutant *aba1-1,abi3-1*, in which ABA action is strongly reduced, if

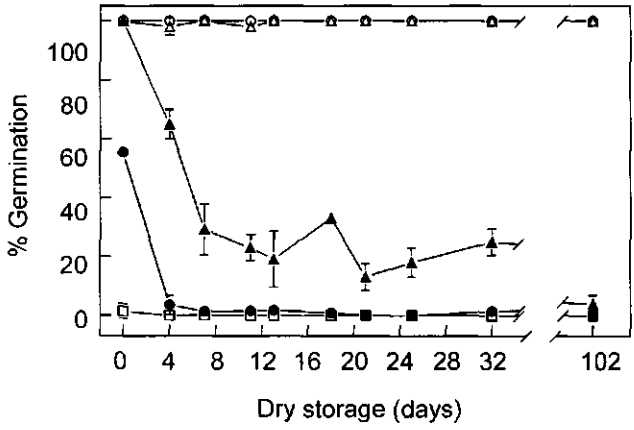

Figure 3.2. Effect of the duration of dry storage (30% RH, 25°C) on germination capacity of seeds from mature siliques. Seeds were wild-type (△), *abi3-1* (○), *abi3-4* (▲), *abi3-5* (●), and *aba1-1,abi3-1* ( ). Data are averages ± SD of triplicate determinations.

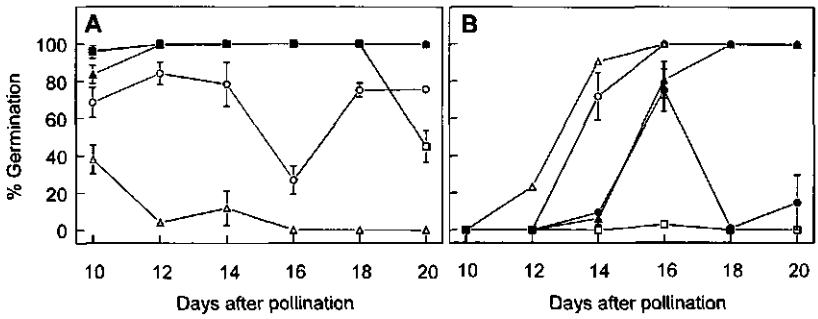

Figure 3.3. Germination capacity of developing *Arabidopsis* seeds. Freshly harvested developing seeds (A) were germinated on water. Dried seeds (B) (48 h, 24°C and 30% RH), were germinated on 100 µM GA_{4+7}. Seeds were wild-type (Δ), *abi3-1* (O), *abi3-4* (▲), *abi3-5* (●), and *aba1-1,abi3-1* (□). Data are averages ± SD of triplicate determinations.

present at all (Koornneef et al., 1989), and the extreme *abi3-3* allele described by Nambara et al. (1992).

We further tested these seeds for survival during dry storage. Seeds from mature dry siliques were stored at 25°C and 30% RH. After different storage periods, seeds were tested for germination in 100 µM GA_{4+7} (Fig. 3.2). Wild-type and *abi3-1* seeds can withstand long periods of dry storage. The double mutant *aba1-1,abi3-1* does not survive desiccation at all, whereas the *abi3-4* and *abi3-5* seeds are intermediate in this respect.

In Figure 3.3, A and B, germination capacities during seed development of freshly harvested and dried mutant seeds are shown and compared with those of wild-type seeds. Seeds of all genotypes except the double mutant *aba1-1,abi3-1* acquire desiccation tolerance, which seems to be transient in the case of *abi3-5* (Fig. 3.3B). The loss of desiccation tolerance in *abi3-5* seeds 16 days after pollination (DAP) might be caused by precocious germination due to the absence of dormancy (Fig. 3.3A). Freshly harvested wild-type seeds do not germinate from 16 DAP onward, because they reach full dormancy at this stage. Of the other genotypes, only *abi3-1* seeds develop some dormancy (Fig. 3.3A). The degree of dormancy of the seeds carrying *abi3* alleles correlates with their ABA-sensitivity (Fig. 3.1). The reduced germination of *aba1-1,abi3-1* double mutant seeds at 20 DAP is caused by dehydration of seeds in the siliques, prior to the germination test.

Carbohydrates

To investigate the role of soluble carbohydrates in the acquisition of desiccation tolerance, we analyzed the carbohydrate composition during seed development of the five genotypes (Fig. 3.4, A-E). Due to the low seed weight, ranging from 14-20

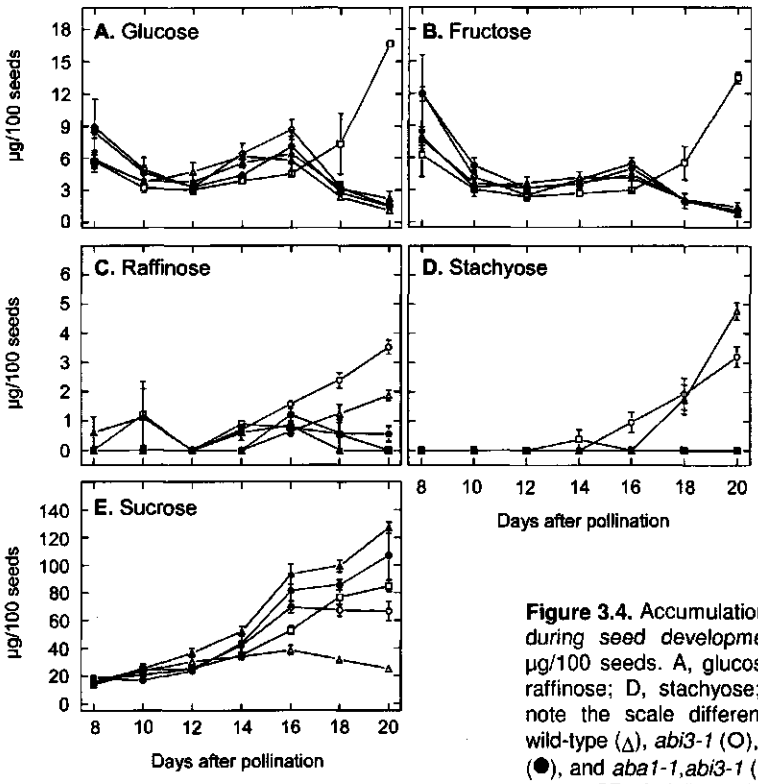

Figure 3.4. Accumulation of carbohydrates during seed development, expressed in µg/100 seeds. A, glucose; B, fructose; C, raffinose; D, stachyose; and E, sucrose; note the scale differences. Seeds were wild-type (△), *abi3-1* (○), *abi3-4* (▲), *abi3-5* (●), and *aba1-1,abi3-1* (□). Data are averages ± SD of triplicate determinations.

µg for dry mature seed, an accurate determination of dry weights during the course of seed development is hardly possible. Thus, carbohydrate content during seed development is expressed in µg/100 seeds. Figure 3.4, A and B, reveal that the amount of glucose and fructose are more or less equal in all genotypes until 16 DAP. From 16 DAP onward, the amount of glucose and fructose strongly increases in *aba1-1,abi3-1* double mutant seeds, whereas all other genotypes show a small decrease in monosaccharide content. Raffinose and stachyose, although present in minor quantities, increase in amount in the later stages of maturation in wild-type and *abi3-1* seeds (Fig. 3.4, C and D). In the *abi3-4, abi3-5* and *aba1-1,abi3-1* double mutant seeds, the amounts of raffinose and stachyose are below the detection limit. Compared with mature wild-type seeds, the different mature mutant seeds have three to five times more sucrose (Fig. 3.4E).

In Table 3.1, the carbohydrate data from Figure 3.4 are summarized and the

Table 3.1. Relative amounts of monosaccharides (glucose + fructose), disaccharide (sucrose) and oligosaccharides (raffinose + stachyose) present in 20-DAP (mature) seeds. Values are calculated from the data in Figure 3.4.

Genotype	Relative amounts of carbohydrates			Ratio mono-/oligo-saccharides	Total sugar content
	Mono-saccharides	Disaccha-rides	Oligo-saccharides		
		%			ng/mgdry weight
wild-type	11.1	77.9	10.9	1.02	55.4
abi3-1	6.3	88.8	4.9	1.28	135.1
abi3-4	4.3	95.4	0.3	15.19	165.5
abi3-5	5.1	94.7	0.2	21.66	209.4
aba1-1,abi3-1	40.2	59.6	0.1	354.12	296.6

ratio of mono- to oligosaccharides in mature seeds has been calculated. These data suggest a correlation between a low mono-/oligosaccharides ratio and desiccation tolerance. However, this correlation was not found for immature, desiccation tolerant developing seeds. Nevertheless, a correlation between the mono-/oligosaccharides ratio and longevity cannot be excluded.

Proteins

We tested whether proteins present in *Arabidopsis* seeds cross-reacted with antibodies raised against ABA- or dehydration-induced proteins of the desiccation-tolerant resurrection plant *Craterostigma plantagineum* and barley embryos (Piatkowski et al., 1990; Bartels et al., 1991). Antibodies raised against pcC6-19 and pcC27-45 cDNA-derived polypeptides overexpressed in *Escherichia coli* were used. The protein encoded by pcC6-19 shows homology with proteins expressed during late embryogenesis in seeds of higher plants. Proteins encoded by pcC27-45 are suggested to be characteristic of *Craterostigma* (Piatkowski et al., 1990). The cDNA clone encoding pG22-69 has been isolated from desiccation-tolerant barley embryos and shows structural homology to NADPH-dependent aldose reductase synthesized in mammals (Bartels et al., 1991). Figure 3.5 shows immuno-western blots of *Arabidopsis* protein extracts from seeds of different developmental stages incubated with the pcC6-19 antibody. It illustrates that in the later stage of seed development, all genotypes, except the *aba1-1,abi3-1* double mutant, synthesize at least one protein that is immunologically related with pcC6-19. With the antibody against the *Craterostigma*-specific protein pcC27-45, no cross-reaction was found (not shown). In the wild-type and *abi3-1* seeds, some cross-reaction is found with

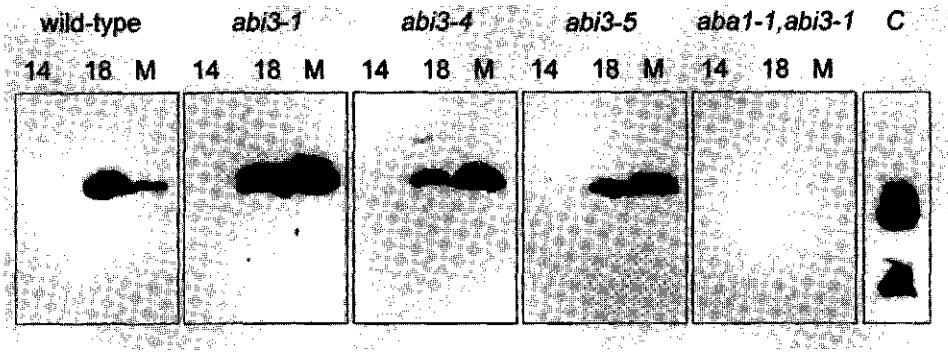

Figure 3.5. Western blot analysis. Protein extracts were prepared from of 14- and 18-DAP and mature (M) intact siliques and incubated with pcC6-19 antiserum. The control lane contains a crude protein extract from dried *Craterostigma* leaves (C). Note that band intensity is not a quantitative measure of protein amount.

antibodies directed against the polypeptide encoded by pG22-69 from barley (not shown).

Discussion

In this study, we introduce two new ABA-insensitive *Arabidopsis* mutants that are affected in the *ABI3* locus, as can be concluded from the complementation tests and map position. In contrast to the *abi1* and *abi2* mutants, the *abi3* mutations specifically affect seed development (Koornneef et al., 1984; Finkelstein and Somerville, 1990; Nambara et al., 1992). ABA is generally known to play a crucial role in seed maturation and acquisition of desiccation tolerance (Koornneef et al., 1989; Black, 1991). Consequently, it can be expected that a comparative study using these mutants can contribute to the understanding of seed maturation and desiccation tolerance.

The capacity of a seed to germinate after being stored in the dry state is often used as a measure of desiccation tolerance. However, storage time and residual moisture content are generally not clearly defined. For practical reasons, we define desiccation tolerance as the capacity of a seed to grow into normal seedlings after a 48-h desiccation period at 25°C and 30% RH. Under these conditions, the water content of the seeds is reduced to approximately 5% of the dry weight. Survival of dry storage for longer periods is covered by the general term longevity.

Comparison of Figure 3.1, in which sensitivity of germination to ABA is tested, with survival of dry storage as shown in Figure 3.2, suggests a positive

correlation between ABA-sensitivity and longevity. In the course of seed development, wild-type seeds are the first to acquire desiccation tolerance, followed by *abi3-1*, *abi3-4*, and *abi3-5* seeds (Fig. 3.3B). The *aba1-1,abi3-1* double mutant and the *abi3-5* seeds acquire a transient desiccation tolerance around 16 DAP (see also Figure 2B in Koornneef et al., 1989). The immediate loss of desiccation tolerance in these two genotypes is possibly due to precocious germination in the siliques caused by the high RH at which plants are grown and the lack of dormancy in these seeds.

The extent of germination of freshly harvested seeds (Fig. 3.3A) in the later stages of development correlates with ABA-insensitivity (Fig. 3.1). The severe ABA-insensitivity also correlates with the absence of chlorophyll breakdown during seed maturation and, according to Nambara et al. (1992), the absence of 12S and 2S proteins. Dormancy develops in wild-type and, to a small extent, in *abi3-1* seeds, whereas it is absent from the new *abi3-4* and *abi3-5* mutant seeds and the *aba1-1,abi3-1* double mutant (Koornneef et al., 1989). These results confirm the earlier established role of ABA in seed development (Karssen et al., 1983; Koornneef et al., 1989). The data further support the essential role of the ABI3 protein as a regulator of seed maturation, which possibly acts in combination with ABA or an ABA-induced protein. The extreme insensitivity to ABA of the *abi3-4* and *abi3-5* alleles further indicates that the inhibitory effect of an excess of exogenous ABA is mediated by this ABI3 protein. The observation that *vp1* mutants are only slightly resistant to ABA (Robichaud et al., 1980) indicates that in this respect the *abi3* mutants differ from *vp1* mutants. In addition, no obvious effects on anthocyanin content are observed in the *abi3* mutants; anthocyanins do not accumulate in seeds of the maize *vp1* mutants (Hattori et al., 1992). However, especially in their effects on seed-specific and ABA-affected characters, the *abi3* and *vp1* mutants are similar, which together with the resemblance of the DNA sequence (Giraudat et al., 1992) suggests overlapping but probably not completely identical functions of these genes.

Many organisms in the dehydrated stage contain large amounts of soluble carbohydrates. Desiccation tolerance in seeds and pollen is associated with the accumulation of sucrose and oligosaccharides like raffinose and stachyose (Amuti and Pollard, 1977; Hoekstra et al., 1989; Leopold, 1990). With the isolation of the *abi3-4* and *abi3-5* mutants, we obtained a series of *Arabidopsis* mutant seeds that differ in the acquisition of desiccation tolerance and longevity (Figs. 3.2 and 3.3B). With the use of these seeds, we examined whether desiccation tolerance correlates with accumulation of certain soluble carbohydrates. Only in the *aba1-1,abi3-1* double-mutant do the amounts of glucose and fructose strongly deviate from those found in wild-type seeds (Fig. 3.4, A and B). Only raffinose and stachyose

accumulate in the desiccation-tolerant wild-type and *abi3-1* seeds, whereas this accumulation is minimal or not detected in the other genotypes (Fig. 3.4, C and D). However, accumulation of raffinose and stachyose is not temporally linked with acquisition of desiccation tolerance (compare Fig. 3.4, C and D, and Fig. 3.3B). The most desiccation-tolerant seeds, wild-type and *abi3-1* have the lowest amounts of sucrose (Fig. 3.4E). Moreover, as illustrated in Table 3.1, the genotypes which have the highest desiccation tolerance (Fig. 3.3B) accumulate the lowest total amounts of soluble sugars in the later part of seed development. The huge accumulation of carbohydrates in desiccation-intolerant tissues, together with the temporal separation of oligosaccharide accumulation and development of desiccation tolerance, leads us to the conclusion that accumulation of carbohydrates as such is not sufficient for acquisition of desiccation tolerance in *Arabidopsis* seeds.

Table 3.1 and Figure 3.2 suggest a correlation between a low mono-/oligosaccharides ratio and longevity. Stable glass formation, which is suggested to be essential for acquisition of desiccation tolerance (Bruni and Leopold, 1991), depends on the mono-/oligosaccharides ratio (Koster, 1991). Consequently, it is suggested that the declining longevity of the mutant seed of *abi3-4* and *abi3-5* could be caused by instable glass formation, eventually expressed as desiccation intolerance in *aba1-1,abi3-1* double mutant seeds.

Besides the direct protective aspects of carbohydrates, accumulation of carbohydrates will contribute to the osmotic potential of the seeds. It has been shown that not only ABA but also osmotic effects are important for normal seed development, acquisition of desiccation tolerance, and prevention of precocious germination (Finkelstein and Crouch, 1986, 1987; Xu et al., 1990). The *aba1-1,abi3-1* double mutant seeds, which strongly accumulate carbohydrates, germinate precociously and synthesize germination-related proteins instead of maturation-related proteins in the later stages of seed development (Meurs et al., 1992). Consequently, the accumulation of carbohydrates in *abi3-4* and *abi3-5* seeds might correlate with precocious germination together with a similar change in protein pattern. However, our results indicate that seeds of all genotypes except the *aba1-1,abi3-1* double mutant synthesize a maturation related protein showing homology to the ABA- and desiccation-inducible protein encoded by cDNA clone pcC6-19, which was isolated from *Craterostigma plantagineum* (Fig. 3.5). Thus, it is not likely that in the *abi3-4* and *abi3-5* mutant seeds a changed protein pattern concomitant with precocious germination is the cause of a transient desiccation tolerance. Rather, the presence of this protein in all genotypes except in the double mutant suggests a correlation with desiccation tolerance. However, in 14 dap seeds, no detectable cross-reaction was found, whereas wild-type and *abi3-1* seeds have acquired nearly full desiccation tolerance at this stage of development. Thus, the

expression of this protein is not temporally related to the acquisition of desiccation tolerance or longevity.

We also found cross-reaction with an antibody against pG22-69 in wild-type and *abi3-1* seeds (not shown). This indicates the presence of a protein in *Arabidopsis* seeds with homology to a functional aldose reductase in barley embryos (Bartels et al., 1991). Aldose reductase is known to be involved in the salt-stress-induced conversion of glucose to sorbitol in mammalian cells and functions in osmoregulation (Perez et al., 1989). However, the presence of sorbitol has not been established in *Arabidopsis* seeds.

Our results show that the presence of endogenous ABA and the absence or negligible amounts of the ABI3 gene product is sufficient to induce desiccation tolerance as found in *abi3-4* and *abi3-5* seeds. Apparently, the endogenous ABA is not sufficient to extend longevity. The different patterns of carbohydrate accumulation in the ABA mutants are indicative of a role of ABA in the regulation of carbohydrate metabolism. The presence of a protein with homology to aldose reductase in the wild-type and *abi3-1* seeds, which are both desiccation tolerant and have an extended longevity, supports this idea. Accumulation of carbohydrates as such does not seem to be a prerequisite for desiccation tolerance. However, the composition of carbohydrates may influence seed longevity.

Materials and methods

Plant material

All genotypes used have been derived from *Arabidopsis thaliana* ecotype Landsberg *erecta* (L*er*, wild-type). The *abi3-1* mutant (isolation number CIV), and the *aba1-1,abi3-1* double mutant (isolation numbers A26 and CIV, respectively) have been described by Koornneef et al. (1982, 1984, 1989). The mutant line SM1 was generated in *abi3-1* seeds by applying 15 mM ethylmethanesulfonate (EMS) for 24 hr. The SM1 mutant was isolated as a dark green M_2 seed, which germinated immediately after harvest. The mutant line 10286 was kindly provided by Dr. L. Conway (University of Pennsylvania, Philadelphia, Pennsylvania, USA) as an M_3 line, segregating for green seeds. It was generated by treating L*er* seeds with 11 mM diepoxybutanol (DEB) for 4 hr. To test allelism, crosses were made between 10286 and SM1 and between 10286 and CIV. The F_1 seeds were sown in petri dishes containing filter paper saturated with 10 mM ABA and incubated at 25°C, with a 16-hr light period. Germination was scored after 7 d. For mapping, crosses were made between SM1 and lines containing the visible markers *hy2* and *gl1*. F_2 populations derived from these crosses were scored for plants with brown, green,

and a mixture of brown and green seeds together with the marker phenotypes.

For plant culture, seeds were sown in petri dishes on water saturated filter paper. The petri dishes were placed in an incubator at 25°C under continuous light (8 W/m^2). After 2 to 4 d, germinated seeds were transferred into soil and cultivated in a greenhouse (18-22°C) with additional light (Philips TLD 58W/84, 16-hr light period).

Seed collection

Mature seeds were harvested from dehydrated siliques. Staging of the developing seeds was performed by tagging individual flowers on the day of anthesis. For the analyses of dormancy, desiccation tolerance, and carbohydrate content, collection of young seeds during development was carried out in a glove box under 100% RH, to avoid possible changes due to moisture loss. Immature seeds were collected by opening unripe siliques using a needle and forceps. Collected seeds were immediately placed on water saturated filter paper or dry filter paper or immersed in 80% methanol, for the analysis of dormancy, desiccation tolerance, and carbohydrates, respectively.

Germination assays

To determine the sensitivity of germination to ABA, 40-80 mature seeds were sown in triplicate in petri dishes containing filter paper soaked with a range of ABA concentrations. The petri dishes were stored for 4 d at 4°C and subsequently incubated in a growth chamber (25°C, 16-hr light period). Germination was scored 7 d after the start of incubation at 25°C. To determine dormancy in developing seeds the contents of at least two siliques (40-60 seeds) were placed in triplicate in petri dishes containing filter paper soaked with water. Germination was scored after a 14-d incubation at 25°C in continuous light. To determine desiccation tolerance in developing seeds the contents of at least two siliques (40-60 seeds) were placed in triplicate in open petri dishes containing dry filter paper, and the petri dishes were placed in an incubator (30% RH, 25°C). After 2 d, the filter paper was soaked with 100 mM GA_{4+7}. Germination was scored after 14 d of incubation at 25°C in continuous light.

Carbohydrate analysis

Approximately 50 seeds were homogenized in 0.5 ml 80% methanol with 25 µg of melezitose as the internal standard. The homogenate was boiled for 15 min at 75°C and the methanol was evaporated under vacuum at 20°C. The dried pellet was taken up in water, and the suspension was centrifuged in an Eppendorf centrifuge (5 min, maximum speed). The supernatant was directly injected in a Dionex HPLC system

(Dionex Corporation, Sunnyvale, California, USA), equipped with a Carbopac PA100 column and a pulse-amperometric detection system. Samples were eluted isocratically with 0.1 N NaOH. Carbohydrates were identified by comparing chromatographs with standards under two different elution protocols.

Protein extraction

For protein extraction, dry mature seeds or intact immature siliques were frozen in liquid nitrogen. Either approximately 3 mg of dry seeds or six siliques were used for one extraction and homogenized in 100 µl of sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% ß-mercaptoethanol, 0.025% bromophenol blue). Proteins were separated on a 12% SDS polyacrylamide gel and electroblotted from the gel to a nitrocellulose membrane. The membranes were probed with rabbit antiserum (1:1000 diluted) raised against ABA-induced proteins from *Craterostigma plantagineum* (Schneider et al., 1993) or aldose reductase from barley (Bartels et al., 1991), and further incubated with anti-rabbit immunoglobulin G peroxidase conjugate (Sigma). The protein antibody complex was detected using the chemiluminescence (ECL) western blotting detection system from Amersham (UK).

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CHAPTER 4

Physiological and biochemical analysis of seed maturation mutants from the *abi3* and *lec* class

Karen M. Léon-Kloosterziel
Adriëtte W. de Visser
Corrie J. Hanhart
Hetty Blankestijn-de Vries
Anton J.M. Peeters
Maarten Koornneef

Summary

A new mutant at the ABI3 locus was isolated by means of a selection for germination on 100 μM abscisic acid (ABA). The phenotype of this mutant allele (abi3-7) was intermediate between the weak abi3-1 and the extreme abi3-4 and abi3-5 alleles. This was demonstrated with respect to ABA-insensitivity of germination, germination rate, and carbohydrate composition. In contrast, the abi3-7 mutant showed a deviant expression of the storage protein gene at2S2 and the LEA genes Em1 and Em6. The extreme abi3 mutants were analyzed at the biochemical and physiological level, together with the lec1, lec2, and fus3 mutants, which show a severely disturbed seed maturation as well. They all have, except lec2, strongly reduced levels of the 12S and 2S seed storage proteins and elevated levels of carbohydrates, in particular sucrose. The lack of desiccation tolerance of these mutants could not be correlated to the absence of specific carbohydrates. The increase in carbohydrate levels is probably an indirect result of the decreased storage compound levels. With the use of double mutants it was shown that the abi3 and lec1 mutations, in contrast to the fus3 mutation, abolished the GA requirement for germination. This may suggest that a dormancy mechanism is based on the induction of a GA requirement by ABA.

Introduction

During seed development, the seed enters a stage of maturation after embryo growth has been completed. The maturation stage is characterized by a number of specific processes that include the accumulation of storage lipids and proteins, and the induction and maintenance of dormancy and desiccation tolerance. At the end of seed maturation water loss and chlorophyll breakdown occur. In *Arabidopsis*, a set of mutants is known that shows dramatic defects in several aspects of seed maturation. The effects of the mutations are strictly confined to seeds and therefore the mutants show a normal wild-type plant phenotype.

The *abi3* (*ABA-insensitive 3*) mutants have been isolated by their abscisic acid (ABA) or uniconazol insensitivity at germination or on the basis of their green seed colour (Koornneef et al., 1984; Nambara et al., 1992; Ooms et al., 1993). The severity of the phenotypes of the different alleles of this locus vary, but the null mutant allele that shows an internal deletion of approximately 1/3 of the gene, produces green seeds that are insensitive to high ABA concentrations, very non-dormant, desiccation intolerant and accumulate reduced amounts of storage proteins (Nambara et al., 1994).

Another class of seed maturation mutants are the *lec* (*leafy cotyledon*) class mutants, which comprise the *lec1*, *lec2*, and *fus3* (*fusca 3*) mutants. These share characteristics with *abi3* such as lack of dormancy and desiccation tolerance and reduced levels of storage compounds. In addition to these features, the *lec* class mutants exhibit excessive anthocyanin accumulation in the embryo and bear trichomes on their cotyledons The *fus3* mutant is sensitive to ABA-inhibition of seed germination (Meinke, 1992; Bäumlein et al., 1994; Keith et al., 1994; Meinke et al., 1994; West et al., 1994). In the *lec1* mutant, radicle emergence is inhibited during culture on ABA medium (Meinke et al., 1994), but with respect to cotyledon expansion *lec1* can be considered as ABA-insensitive (Parcy et al., 1997)

At present, only the *ABI3* gene has been cloned (Giraudat et al., 1992). It is a homologue of the maize *VP1* gene and considered to be a seed-specific transcription factor. Northern blot analyses of developing mutant seeds have shown that *ABI3* is involved in regulating many, but not all seed maturation genes from different classes (Parcy et al., 1994). From these studies and also from the analysis of plants that ectopically express the *ABI3* gene, it is concluded that the *ABI3* gene is an important, but not the only factor in parts of the signal transduction cascade of ABA as it acts in seeds. It is also clear from the mutant phenotypes that in addition to *ABI3* other genes such as *LEC1*, *LEC2*, and *FUS3* are required for normal seed

maturation.

We isolated several additional mutant alleles of the *ABI3* locus. The physiological analysis of *abi3-4* and *abi3-5* is described by Ooms et al. (1993). In addition, a new *lec1* allele was isolated in a transposon tagging experiment. The goal of the experiments described in this chapter was the comparison of the different *abi3* alleles and the *lec* class mutants, including the new *lec1* allele. This analysis with respect to the accumulation of storage compounds and germination behaviour was performed in order to assess the specific roles of the different genes in the physiology of seed maturation.

Results and discussion

Comparison of different *abi3* alleles

Several mutant screens, performed to isolate mutants affected in seed maturation, yielded *abi3* mutants. A comparative physiological analysis of the *abi3-1*, *abi3-4*, *abi3-5*, and *abi3-7* mutants, differing in severity of the mutant phenotype, was performed in order to study the regulatory function of the *ABI3* gene and examine the correlation between the different mutant traits. The *abi3-7* allele has not been described before and was selected from mutagenized *abi3-1* seeds on the basis of its ability to germinate on 100 µM ABA.

The ABA dose-response curve of the wild-type, the *abi3-1*, *abi3-4*, *abi3-5*, and *abi3-7* mutants (Fig. 4.1) showed that *abi3-5* was the most extreme allele and that *abi3-7* was an allele with an intermediate ABA-insensitivity. This allele also showed an intermediate behaviour with respect to germination rate (Fig. 4.2). Seeds

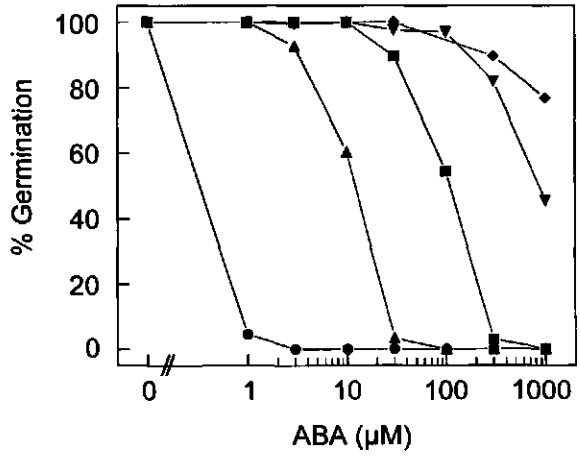

Figure 4.1. ABA-sensitivity of different *abi3* mutants. Germination on various ABA concentrations of the *abi3-1* (▲), *abi3-4* (▼), *abi3-5* (◆), and *abi3-7* (■) mutants and the wild-type (●). Percentages are averages of triplicates.

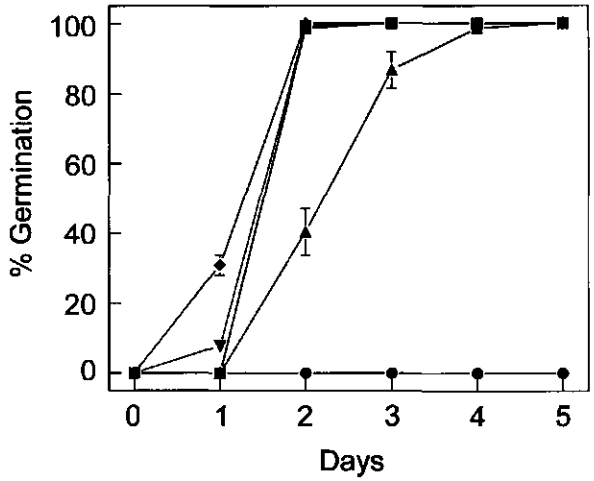

Figure 4.2. Germination rate of freshly harvested seeds of the *abi3-1* (▲), *abi3-4* (▼), *abi3-5* (◆), and *abi3-7* (■) mutants and the wild-type (●). Percentages are averages of triplicates ± SE.

of the *abi3-4* and *abi3-5* mutants were green and *abi3-1* and *abi3-7* seeds had the normal brown colour.

The *abi3* mutants have been subjected to a carbohydrate analysis (Table 4.1). The *abi3* mutants had elevated levels of carbohydrates, mainly due to an elevated level of sucrose. The *abi3-4* mutants had the lowest relative mono- and oligosaccharide amounts, *abi3-1* the highest and *abi3-7* intermediate, correlating with the degree of ABA-resistance. This shift in carbohydrate composition corresponds with the results described by Ooms et al. (1993), where the carbohydrate composition was investigated in correlation with desiccation tolerance. It was hypothesized that not the amount of oligosaccharides as such but the ratio of monosaccharides to oligosaccharides could account for desiccation tolerance in the wild-type, since this ratio increased 20-350 fold in desiccation intolerant (*abi3-5* and *aba1-1,abi3-1*, respectively) seeds. In the data presented here, only a slight increase in monosaccharide/oligosaccharide ratios can be seen in the desiccation intolerant *abi3-4* mutant.

In the severe *abi3-3* allele, no expression of the 2S storage protein clone at2S2 could be detected (Nambara et al., 1992). The *abi3-1*, *abi3-3*, *abi3-4*, and *abi3-7* mutants were included in a more detailed analysis of at2S1 and at2S2 expression, determining expression in a slot blot and *in situ*. An *in situ* hybridization experiment using the at2S1 clone confirmed that this gene was not expressed in *abi3-3*, but signal was detected in *abi3-1* and *abi3-7* seeds. A slot blot analysis using at2S2 as a probe showed that the levels of at2S2 mRNA in *abi3-7* siliques were intermediate between levels in *abi3-1* and in *abi3-4* or *-5* siliques (Da Silva Conceição, 1993).

In the various aspects of the mutant phenotype, the *abi3-7* mutant was an

Table 4.1. Relative amounts of monosaccharides (glucose and fructose), disaccharide (sucrose) and oligosaccharides (raffinose and stachyose) in dry mature seeds of the *abi3* mutants and the wild-type L*er*.

Genotype	Relative amounts of carbohydrates			Ratio mono-/oligo-saccharides	Total content
	Mono-saccharides	Disaccha-rides	Oligo-saccharides		
		%			µg/seed
L*er*	2.1	79.5	18.3	0.11	0.7
abi3-1	1.9	86.5	11.6	0.16	1.2
abi3-7	1.4	90.2	8.4	0.17	1.4
abi3-4	1.2	96.6	2.3	0.53	1.9

allele of intermediate severity between *abi3-1* and the severe alleles, *abi3-3*, *-4*, and *-5*. This means that the functions of the *ABI3* gene as can be deduced from the severity of the phenotypic effects, are not qualitatively dependent from the way and position the gene is mutated. However, it may be possible that specific domains in the *ABI3* gene regulate different specific target genes. The observation has been made in an *in situ* hybridization experiment that *abi3-7* seeds, in contrast to both the wild-type and *abi3-1*, showed expression of at2S2 in the shoot apical meristem (Da Silva Conceição, 1993). Also with respect to expression of the LEA genes *AtEm1* and *AtEm6* (Gaubier et al., 1993), this mutant was deviating from the other alleles. Immunoblots showed the presence of the Em1 and Em6 protein in seeds of the *abi3*-1 mutant. In *abi3*-4 and *abi3*-5 seeds, these proteins were absent, whereas in *abi3*-7 seeds Em1, but not Em6, was detected (Bies, 1997). Of the maize homologue of *abi3*, *vp1*, alleles are known in which specific functions are affected. These exhibit anthocyanin accumulation, but not vivipary, indicating that in this case different functions can be assigned to specific domains in the gene (McCarty et al., 1989).

Biochemical characterization of seed maturation mutants

A characteristic aspect of seed maturation is the accumulation of storage proteins. In Cruciferae such as *Arabidopsis*, two classes of abundant storage proteins are present, the 12S and 2S proteins (Heath, 1986). Figure 4.3 shows the protein patterns of mature seeds of different seed maturation mutants in which the position of the storage proteins is indicated. The *abi3-4* and *lec1-1* mutants lacked the 2S proteins and showed reduced amounts of the 12S proteins. The *lec1-3* and *fus3-2*

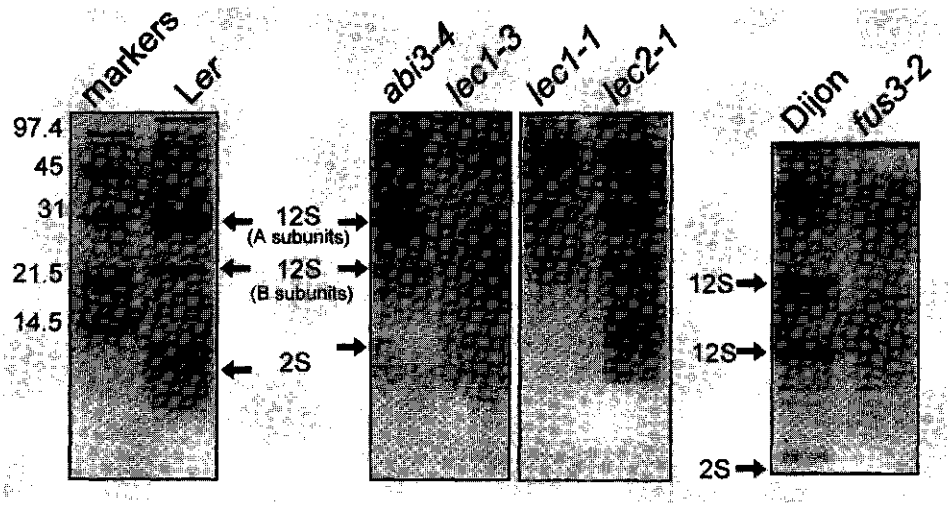

Figure 4.3. Polyacrylamide-gelelectroforesis of proteins extracted from mature seeds of the *abi3-4*, *lec1-3*, *lec1-1*, *lec2-1*, and *fus3-2* mutants and of the *Ler* and Dijon wild-types.

mutants showed a similar pattern, a negligible amount of 12S and 2S proteins was present in these mutants. In contrast to this, seeds of the *lec2-1* mutant showed near wild-type levels of storage proteins. This is in accordance with results of ultra-structural analysis of *lec2-1* that showed that protein bodies are still present in *lec2* seeds (Meinke et al., 1994).

Seeds contain a considerable amount of soluble carbohydrates. In Table 4.2 the results of carbohydrate analysis of several seed maturation mutants is given. All seed maturation mutants had a higher total carbohydrate content than the wild-types and it was mainly a rise in sucrose levels that accounted for this increase. In these mutants the relative amounts of oligosaccharides were lower than in the wild-types, whereas the relative amounts of monosaccharides were very variable. The *lec2-1* mutant was the less extreme mutant with respect to alterations in carbohydrate composition.

The presence of certain sugars is often related to desiccation tolerance. Desiccation-induced injury involves mainly membrane damage and subsequent leakage of cytoplasmic compounds. Sugars can be involved in the protection of membranes in the dried state and play a role in the vitrification (glass formation) within the cytoplasm in order to prevent crystallization upon drying (Leprince et al., 1993). The relative amounts of oligosaccharides in *lec1-1*, *lec1-3*, and *fus3-2* mutant seeds were substantially lower than in the corresponding wild-types and

Table 4.2. Relative amounts of monosaccharides (M, glucose and fructose), disaccharide (D, sucrose), and oligosaccharides (O, raffinose and stachyose) in mature seeds of seed maturation mutants and wild-types.

Genotype (corresponding wild-type)	Relative amounts			Total content	Ratio M/O	Ratio O/D
	M	D	O			
		%		µg/seed		
abi3-4 (Ler)	4.3	93.8	1.9	1.1	2.26	0.02
lec1-3 (Ler)	10.3	83.8	5.8	0.9	1.77	0.07
lec1-1 (Ws)	3.5	92.4	4.1	1.2	0.85	0.04
lec2-1 (Ws)	7.2	77.3	15.6	0.9	0.46	0.20
fus3-2 (Dijon)	3.1	90.2	6.7	1.2	0.46	0.07
Ler	8.0	77.6	14.4	0.7	0.56	0.19
Ws	5.6	70.5	23.9	0.7	0.23	0.34
Dijon	3.9	81.1	15.0	0.7	0.26	0.18

even lower in the *abi3-4* mutant. However, in *lec2-1* seeds the relative amount of oligosaccharides was not dramatically lowered. This may suggest a correlation between the amount of oligosaccharides and desiccation tolerance since *lec2-1* is, in contrast to the other mutants, usually desiccation tolerant. Only the distal end of *lec2-1* cotyledons become deflated at seed maturity, leading to germinable seeds (Meinke et al., 1994). However, this correlation is not perfect because the most desiccation intolerant mutants *lec1-1*, *lec1-3*, and *fus3-2* contain more oligosaccharides than *abi3-4*, which can survive at least several days of dry storage. In the *aba1-1,abi3-1* double mutant the oligosaccharide content is also lower than in the wild-type (Ooms et al., 1993). A causal relationship is questionable because wild-type seeds become desiccation tolerant earlier in development than the occurrence of the rise in oligosaccharide levels (Ooms et al., 1993). The mono/oligosaccharide ratio can be important for stable glass formation (Koster, 1991). In the mutants analyzed here, the strongly elevated mono/oligo ratio that was found in the *aba1-1,abi3-1* double mutant was not observed (Table 4.2). Other authors have suggested that the oligosaccharide/sucrose or oligosaccharide/total sugar ratio accounts for desiccation tolerance since oligosaccharides would enhance the assumed protective effect of sucrose by limiting crystallization (Lin and Huang, 1994). The desiccation intolerant seed maturation mutants analyzed here had a low oligo/sucrose ratio in common with recalcitrant tree seeds (Table 4.2; Lin and Huang, 1994).

The higher carbohydrate levels can be a consequence of the lower levels of storage lipids and proteins. Ultrastructural analysis of *lec1-1* and *fus3-2* seeds has shown that these mutants have less lipid and protein bodies and more starch granules (Meinke, 1992; Keith et al., 1994; Meinke et al., 1994). The *fus3-2* mutant has lowered lipid and storage protein content in seeds (Bäumlein et al., 1994). The total net import of assimilates into the seeds was found to be unaffected in the *aba1-1,abi3-1* double mutant, in which also important maturation programs are abolished. However, the distribution into the various storage compounds was different: the seeds accumulated less neutral lipids and more soluble carbohydrates and starch (De Bruin, 1993). This seems also to be the case in the mutants analyzed here. The observation that the sugar composition is more deviating from the wild-type in *abi3-4* seeds than in seeds of the *lec* mutants may also indicate that *ABI3* is more important in the control of storage compound accumulation, whereas the *LEC* genes are relatively more important in desiccation tolerance.

Dormancy and germination

The seed maturation mutants studied here are characterized by an absolute lack of seed dormancy. Sometimes they even show vivipary (germination within the silique) and germination is in general faster than in the wild-type. Most ABA-deficient and -insensitive mutants show a lack of dormancy together with a reduced gibberellin (GA) requirement, as can be concluded to their insensitivity to GA biosynthesis inhibitors (Nambara et al., 1991; Léon-Kloosterziel et al., 1996b). To investigate if a GA requirement for germination depends on the presence of dormancy induced by ABA, we analyzed the GA requirement in other non-dormant seed maturation mutants. For this, double mutants were constructed between the GA-deficient mutant *ga1-3* and the seed maturation mutants *lec1-3, fus3-2,* and *abi3-5*, respectively. The germination capacity of seeds during development was determined by excising seeds from siliques at different stages of development and incubate them on agar plates. The germination curves in the course of development are given in Figure 4.4. When seeds are incubated on filter paper, fresh, mature L*er* seeds exhibit dormancy (Léon-Kloosterziel et al., 1996a), but in the present experiments, where seeds are germinated on agar medium, germination was 100%. Germination of 17 days after pollination (DAP) seeds from the same experiment on filter paper was 1% after 1 week of incubation and 50% after 2 weeks. Apparently, the agar medium on which germination was tested had a promotive effect on germination. The wild-type seeds (L*er* and Dijon) nevertheless germinated somewhat slower than the mutant seeds (not shown). Seeds of the *ga1-3* mutant in

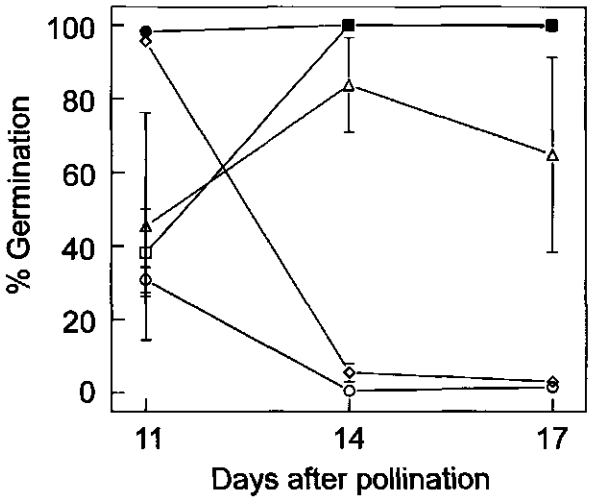

Figure 4.4. Germination of developing seeds of the *ga1-3* (○) mutant, the *ga1-3,lec1-3* (□), *ga1-3,abi3-5* (△), and *ga1-3,fus3-2* (◇) double mutants, and the wild-type L*er* (●). The seeds were removed from the siliques at different stages during development and germinated on water. Percentages are averages of triplicates ± SE.

the later stages did not germinate at all and neither did the *ga1-3,fus3-2* double mutant seeds. The *ga1-3,abi3-5* and *ga1-3,lec1-3* double mutant seeds were capable of germination in spite of their GA deficiency. Nambara et al. (1992) also showed this for *ga1-2,abi3-3* seeds.

Near-mature seeds at 17 DAP, sown on a range of GA concentrations, indicated that *ga1-3,fus3-2* behaved like the *ga1-3* single mutant, and that *ga1-3,lec1-3* seeds were able to germinate without GA and that *ga1-3,abi3-5* had an intermediate GA requirement (Fig. 4.5). From these data it can be concluded that *lec1-3* does not need GA for germination, whereas *fus3-2* does. The low GA requirement of the *abi3* and *lec1* mutants correlates with the ABA-insensitivity of these mutants. In the *fus3* mutant, the lack of dormancy does not seem to be associated with reduced GA requirement. Another reduced dormancy mutant (*rdo1*) does not show this association either (Léon-Kloosterziel et al., 1996a).

The absence of GA requirement for germination predicts that such mutants are insensitive to inhibitors of GA biosynthesis such as paclobutrazol. The germination of 17 DAP seeds in the presence of paclobutrazol was determined and is shown in Figure 4.6. The *abi3-5* and *fus3-2* mutants were insensitive to this inhibitor up to 100 μM; *lec1-3* mutant seeds were insensitive as well, but to a lesser extent. The paclobutrazol insensitivity of *fus3-2* seeds is remarkable, because 10-12 DAP *fus3-3* seeds have been reported to be as sensitive to the GA inhibitor uniconazol as the wild-type (Keith et al., 1994). It also contradicts the observation that the *ga1-3,fus3-2* double mutant is not able to germinate. However, it might be possible that the GA requirement is dependent on the stage of seed development. Seventeen DAP *fus3-2* seeds may already be so much advanced in the germination

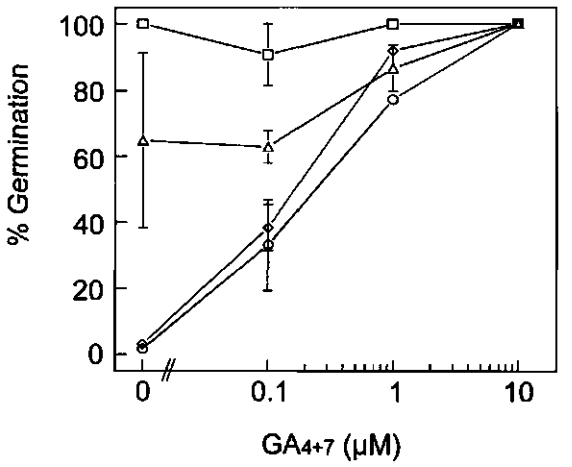

Figure 4.5. GA requirement for germination. Germination on various concentrations of GA_{4+7} of mature seeds of the *ga1-3* (○) mutant, and the *ga1-3,lec1-3* (□), *ga1-3,abi3-5* (△), and *ga1-3,fus3-2* (◊) double mutant. Percentages are averages of triplicates ± SE.

process that GA biosynthesis has already occurred and/or that the GA requirement for seed germination has been satisfied. Nambara et al. (1991) showed that germinating wild-type seeds can only be inhibited by GA inhibitors up to 24 hr of imbibition, which is 12-24 hr before root protrusion is observed. Indications exist that in the very non-dormant *abi3-3* mutant the germination process is already initiated during seed development (Nambara et al., 1995). Testing the paclobutrazol resistance during seed development will be required to confirm this hypothesis.

A complication with the interpretation of these data is that *fus3-2* is in a different genetic background (Dijon) than *abi3-5* and *lec1-3* (both L*er*). It cannot be excluded that these ecotype genetic differences interfere with the GA requirement during germination. However, the exactly similar paclobutrazol sensitivity of both ecotypes does not suggest this.

Concluding remarks

Mutations in the *ABI3*, *LEC1*, *LEC2*, and *FUS3* genes result in part in similar phenotypes; the obvious differences are that *ABI3* is involved in the ABA signal transduction pathway and that the *LEC* class genes are involved in the timing of seed development stages and/or the determination of embryonic organ identity. In this respect, the typical anthocyanin accumulation in the *lec* class mutants is not well understood. It is clear from the mutant phenotypes that *LEC1* and *FUS3* have closely related functions. Double mutants of *lec1* and *fus3* with *abi3* showed a more severe phenotype than the monogenic mutants (Meinke et al., 1994; Parcy et al., 1997). This indicates that the corresponding genes are not active in independent regulatory pathways.

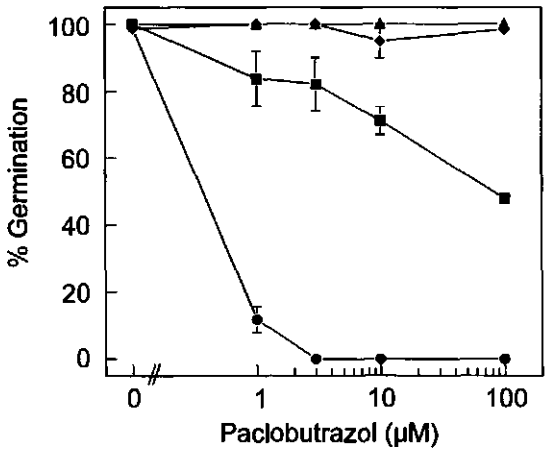

Figure 4.6. Paclobutrazol sensitivity of seed maturation mutants. Germination on various paclobutrazol concentrations of mature seeds of the *lec1-3* (■), *fus3-2* (◆), and *abi3-5* (▲) mutants and of the wild-type Dijon (●). The germination of the wild-type Ler (not shown) was similar to the germination of Dijon. Percentages are averages of triplicates ± SE.

The *ABI3* and *LEC* genes have their specific functions that partly overlap. This can be concluded from the effects on expression of specific seed maturation program genes, such as those encoding storage proteins and lipids, together with ABA-responsive, or dehydration responsive genes. In the *abi3* mutant it has been shown that many, but not all of these different classes of genes are downregulated (Parcy et al., 1994). A much lower number of genes have been studied in the *lec1* and *fus3* mutants, showing that these the *LEC1* and *FUS3* genes control partly common and partly separate genes (West et al., 1994). A more extensive examination of gene expression of seed maturation and germination specific genes in the complete set of mutants, will give more information about the control of gene expression programs during seed maturation.

Our biochemical studies did not reveal any striking quantitative differences between these mutants with respect to carbohydrate and overall protein composition. Gene expression studies with several storage protein genes, however, can give details about the way storage proteins are affected. The *lec1* and *fus3* mutants differ in GA requirement. The *fus3* mutation most likely does not alleviate the GA requirement normally displayed by *Arabidopsis* seeds, whereas *lec1* mutant seeds show a lowered GA requirement, like the *abi3* and ABA-deficient mutants. The fact that *lec1* is also ABA-insensitive (Parcy et al., 1997) may suggest that a dormancy mechanism is based on the induction of a GA requirement by ABA. On the contrary, it might be possible that the GA requirement in *fus3* depends strictly on the stage of maturation. In this way, *fus3* is mainly heterochronic, i.e. that in mutant seeds maturation prematurely switches into germination (Keith et al., 1994). This switch is probably not absolute, since the studies of gene expression showed

that some late embryogenesis genes are still expressed in e.g. *abi3* (Parcy et al., 1994). It might be possible that in such mutants the maturation and germination specific pathways show some overlap. The different maturation mutants may differ in either the timing and/or the specificity of the genes that they control.

Material and methods

Plant material

Plants were grown in an air-conditioned greenhouse (18-23°C) with additional light in winter (Philips HPI-T/400W; 16-hr photoperiod) or in a climate chamber (22°C, 16-hr photoperiod, Philips TL57 and incandescent bulbs).

The mutants used came from various sources. The isolation of the *abi3-1* mutant (isolation no. CIV) has been described before by Koornneef et al. (1984) and of the *abi3-4* and *abi3-5* mutants by Ooms et al. (1993).

The *abi3-7* mutant originated from the mutagenesis experiment described by Ooms et al. (1993), and was isolated in a screen for ABA-insensitivity at germination on agar plates containing Murashige-Skoog macro- and micro elements, 2% (w/v) saccharose, 0.7% (w/v) agar and 100 µM ABA. Mutagenized *abi3-1* (Landsberg *erecta* (L*er*) background) seeds that germinated on these plates were transferred to agar plates without ABA and after 2 weeks planted in soil in the greenhouse. The selected plants were re-tested for ABA-insensitivity by sowing them in petri dishes containing filter paper (Schleicher and Schuell no. 595) saturated with 100µM ABA in water. One line that germinated on this ABA concentration was crossed with the wild-type L*er*. An F_3 line derived from this cross that germinated 100 % on 100 µM ABA on filter paper was selected. A cross of this line with *abi3-5* gave non-dormant greenish F_1 seeds. This indicated that the mutant was allelic to *abi3*; the new mutant was designated *abi3-7*.

The *lec1-1* and *lec2-1* mutants (in Wassielewskia (Ws) background) were kindly provided by Dr. Meinke (Oklahoma State University, Stillwater, Oklahoma, USA) and the *fus3-2* mutant (in Dijon background) by Dr. Miséra (Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany).

The *lec1-3* mutant was isolated from a transposon population. These L*er* lines containing the En/I transposable element system from maize were constructed by Aarts et al. (1995). Among 400 plants, of which immature siliques were scored for the presence of anthocyanin accumulating seeds, one mutant was isolated. This new mutant was stable (no transposase present) and was tagged because re-

introduction of the transposase gene gave rise to a high frequency of reversion. F_1 seeds from a cross between this mutant and a *lec1* mutant showed the mutant phenotype; therefore it was designated *lec1-3*.

Double mutants with *ga1-3* were constructed by crossing the *abi3-5*, *lec1-3*, and *fus3-2* mutants with the GA-deficient deletion mutant *ga1-3* (Sun et al., 1992). F_2 seeds derived from these crosses were germinated on 10^{-5}M GA_{4+7} (ICI). F_2 plants that showed dwarfism were selected and sprayed twice a week with 10^{-5}M GA_{4+7} in order to obtain proper seed set. GA-deficient plants segregating for green (*abi3-5*) or anthocyanin accumulating (*lec* and *fus3*) seeds were selected.

Homozygous *lec1-3*, *fus3-2*, *ga1-3,lec1-3*, and *ga1-3,fus3-2* plants were obtained by removing mutant seeds from immature siliques from plants, heterozygous for the maturation mutation, and germinate these on agar plates with half strength Murashige-Skoog macro- and micro elements and 0.7% agar. After 1 week incubation on the agar plates, the seedlings were transferred to soil.

Protein analysis

Plants were grown in the climate chamber and flowers tagged at the day of anthesis. Seeds from 6 siliques were taken out at 20 days after pollination (DAP), homogenized in acetone at 4°C to remove pigments and centrifugated. The pellet was extracted with extraction buffer (63 mM Tris pH 7.8, 0.5 M NaCl, and 0.07% (w/v) ß-mercaptoethanol) at 4°C and centrifugated. The supernatant was mixed with an equal volume of sample buffer (63mM Tris pH 7.8, 2% (w/v) SDS, 20% (w/v) glycerol, 6% (w/v) ß-mercaptoethanol, and 0.2 mM bromophenolblue) and stored at -20°C. The samples were run in a 12% polyacrylamide in 375 mM Tris pH 8.9 gel and stained with Coomassie Brilliant Blue.

Carbohydrate analyses

For analysis of the *abi3* mutants, 75 mature, dry seeds were extracted in duplicates and analyzed as described before (Ooms et al., 1993). For analysis of the *abi3* and *lec* class mutants, plants were grown in the climate chamber and flowers tagged at the day of anthesis. For each sample, 50 seeds were removed from 20 DAP siliques in triplicate, extracted and analyzed as described before (Ooms et al., 1993).

Germination experiments

Seeds were harvested from dry siliques and sown on filter paper (Schleicher & Schuell no. 595) in petri dishes saturated with water or solutions of ABA or paclobutrazol in water. The petri dishes were placed in a climate room (21°C, continuous light) and germination scored at regular intervals. For the ABA and

paclobutrazol germination experiments, the dishes were placed in the cold room (4°C) for 4 d (paclobutrazol: 7 d) and subsequently incubated in a climate room (25°C, 16-hr photoperiod) and germination scored after 6 d (paclobutrazol: 7 d). For the determination of germination of immature seeds, plants were grown in the climate chamber. GA-deficient mutants were sprayed twice a week with 10^{-5}M GA_{4+7}. Flowers were tagged at the day of anthesis. Siliques of different stages were opened by cutting, and the seeds transferred to agar plates with half strength Murashige-Skoog macro- and micro elements and 0.7% agar. To test the effect of GA, filter-sterilized GA_{4+7} (ICI) was added to this medium. The agar plates were incubated in a climate room (25°C, 16-hr photoperiod) and germination scored after 7 d and after 14 d.

Acknowledgements

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CHAPTER 5

Isolation and characterization of abscisic acid-deficient *Arabidopsis* mutants at two new loci

Karen M. Léon-Kloosterziel
Marta Alvarez Gil
Gerda J. Ruijs
Steven E. Jacobsen
Neil E. Olszewski
Steven H. Schwartz
Jan A.D. Zeevaart
Maarten Koornneef

Summary

Novel Arabidopsis mutants with lowered levels of endogenous abscisic acid (ABA) were isolated. These were selected in a screen for germination in the presence of the gibberellin biosynthesis inhibitor paclobutrazol. Another mutant was isolated in a screen for NaCl tolerance. The ABA-deficiency was caused by two monogenic, recessive mutations, aba2 and aba3, that were both located on chromosome 1. The mutants showed a phenotype that is known to be characteristic for ABA-deficiency: a reduced seed dormancy and excessive water loss, leading to a wilty phenotype. Double mutant analysis, combining different aba mutations indicated the leaky nature of the mutations.

Introduction

The plant hormone abscisic acid (ABA) is a sesquiterpenoid molecule that is derived from xanthophyll carotenoids (C_{40}) via the C_{15} intermediate xanthoxin (Zeevaart and Creelman, 1988; Taylor, 1991). Biochemical analysis of mutants that are deficient in ABA has facilitated the elucidation of the biosynthetic pathway of ABA in higher plants. Most of the mutants have been isolated on the basis of their wilty phenotype or on the basis of seed dormancy characteristics (Koornneef, 1986). In maize, viviparous seeds are easy to identify on cobs segregating for this trait (Robertson, 1955). In *Arabidopsis*, *aba1* mutants were identified among the revertants of non-germinating gibberellin-deficient mutants (Koornneef et al. 1982). In addition, mutants at the *abi1* and *abi2* loci are wilty mutants with a reduced seed dormancy but with normal or enhanced ABA levels (Koornneef et al., 1984). These mutants were shown to be relatively insensitive to the inhibiting effect of ABA and apparently have a defect in ABA response. For most of the ABA-deficient loci the biosynthetic defect is known. Maize *viviparous* (*vp*) mutants have an early block in the biosynthesis of carotenoids (Neill et al., 1986). The *Arabidopsis aba1* mutant (Duckham et al.; 1991, Rock and Zeevaart, 1991) and the *Nicotiana plumbaginifolia aba2* mutant (Marin et al, 1996) are affected in the conversion of zeaxanthin to antheraxanthin and in the subsequent conversion to all-*trans*-violaxanthin, the direct C_{40} precursors of ABA. The tomato *not* mutant and the pea *wil* mutant may be affected in the cleavage of 9'-*cis*-neoxanthin to xanthoxin, but to date this has not been convincingly proven (Taylor, 1991). A large number of mutants are known that affect the last step in ABA biosynthesis, that is, the conversion from ABA-aldehyde to ABA. These include *flc* and *sit* in tomato, *dr* in potato, *aba1* in *N. plumbaginifolia*, and *nar2a* in barley (Leydecker et al., 1995; Taylor, 1991)

Shortly after its discovery, ABA was thought to be mainly a growth inhibitor, but it has become clear that it is involved in several important physiological processes. The phenotype of ABA-biosynthetic mutants indicates its function, for example, failure of stomatal closure leads to the wilty phenotype. The possible mechanisms by which ABA works on the ion fluxes associated with stomatal closure have been reviewed by MacRobbie (1991). Another aspect is the involvement of ABA in stress adaptation. Many genes have been isolated that show expression in correlation with the occurrence of ABA and/or stress like osmotic stress or cold. Studies on the regulation and expression of these genes make clear that ABA is involved in at least part of the complex regulation of stress tolerance (Chandler and Robertson, 1994). The involvement of ABA in stress tolerance is also indicated by mutant analyses. ABA-biosynthetic mutants of *Arabidopsis* do not

develop freezing tolerance (Gilmour and Tomashow, 1991; Heino et al., 1990) and drought resistant root structures (Vartanian et al., 1994).

The involvement of ABA in the induction of dormancy has been demonstrated by studies on ABA-deficient mutants in *Arabidopsis* (Karssen et al., 1983) and tomato (Groot et al., 1992). In plants that are homozygous for both the *aba1-1* and the *abi3-1* mutation, or for severe alleles of *abi3*, dormancy as well as other aspects of seed maturation are abolished. These include the accumulation of storage proteins and the development of desiccation tolerance (Koornneef et al., 1989; Nambara et al., 1992; Ooms et al., 1993).

To date, no mutants of *Arabidopsis* that affect other loci than the *ABA1* locus have been reported, whereas mutants at several other steps of the ABA biosynthesis are known in other species. The present paper describes the isolation and characterization of mutants at additional loci in *Arabidopsis* that result in ABA-deficiency, by means of selection on a GA biosynthesis inhibitor.

Results

Genetic analysis

EMS-mutagenized *Arabidopsis* M_2 seeds were screened for germination in the presence of the GA biosynthesis inhibitor paclobutrazol (Jacobsen and Olszewski, 1993). This screen yielded 22 mutants with a "wilty" phenotype: the plants showed a tendency to wilt, especially in low relative humidity, were slightly darker green and less vigorous than wild-type plants. Allelism tests were performed among the new mutants mutually and with the wilty *aba1* mutant. Of these wilty mutants, 10

Table 5.1. Segregation of mutant phenotypes in F_2's derived from crosses of mutant lines to the wild type (WT).

Cross	Segregating locus	No. of plants		$\chi^{2\,a}$
		Total	Wilty	
J11 x WT	*ABA1/aba1-5*	141	38	0.29
J14 x WT	*ABA2/aba2-1*	146	30	1.54
J12 x WT	*ABA2/aba2*	143	39	0.39
J25 x WT	*ABA3/aba3-1*	146	34	0.23
J212 x WT	*ABA3/aba3*	134	32	0.09

[a] χ^2 calculation for expected ratio of 3:1

Table 5.2. Estimates of recombination percentages with standard errors of the *aba* loci and morphological marker loci on chromosome 1.

Marker	Population*	Rec.%	SE	Marker	Population*	Rec.%	SE
aba2 - ch1	F_3R	7.9	1.5	*aba3 - alb1*	F_3R	6.1	3.0
aba2 - ap1	F_2R	20.4	4.8	*aba3 - dis1*	F_3R	3.7	2.4
aba2 - gl2	F_2R	32.3	4.4	*aba3 - dis1*	F_2R	12.7	4.4
aba2 - th1	F_2R	34.9	6.0	*aba3 - an*	F_2R	17.4	4.4
				aba3 - an	F_2C	15.7	2.6
				aba3 - cer1	F_2R	18.3	5.8
				aba3 - th1	F_2R	23.0	6.5

* Data from F_2 and F_3 populations in repulsion (R) or coupling (C) phase.

were allelic to the *aba1* mutant, including the line J11. The other lines represented two new complementation groups; one group of three lines including J12 and J14 and the other group consisting of nine lines, including lines J25, J210 and J212. The segregation of the wilty phenotype in the F_2s derived from crosses between the wild-type and the mutants fitted to a 3:1 ratio, indicating that the wilty trait was controlled by single recessive mutations (Table 5.1). The expectation that paclobutrazol-resistant, wilty mutants are ABA-deficient was confirmed by the finding of lowered ABA levels in the plant (see below). Therefore the two new loci were named *ABA2* and *ABA3*. No allele numbers could be assigned to all members of the allelic groups because they came from the same bulked M_2 seed stock. The J11, J14, and J25 lines will be used as representative alleles and have been named *aba1-5*, *aba2-1*, and *aba3-1*, respectively. An additional mutant (line CB2a-9), was found in a screen aimed at the isolation of NaCl tolerant mutants applied to γ-irradiation-mutagenized seeds. The selected seedlings reached the green cotyledon stage quickly, whereas control seeds did not germinate or seedlings remained yellow on culture medium with 200 mM NaCl. After transfer to the greenhouse the mutant appeared to be wilty. Allelism tests revealed that CB2a-9 was allelic to *aba3* and it was named *aba3-2*.

The *aba2* and *aba3* mutations were both located on chromosome 1. Close linkage was found between *aba2* and the morphological marker *ch1*, and *aba3* was found to be tightly linked to *alb1* and *dis1*. The recombination percentages between *aba2*, *aba3*, and the morphological markers used are given in Table 5.2 and map positions of *aba2* and *aba3* resulting from these recombination percentages are shown in Figure 5.1.

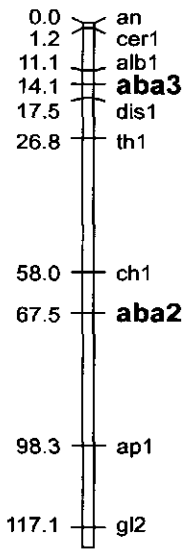

Figure 5.1. Genetic map of the *Arabidopsis* chromosome 1 with the map positions of *aba2* and *aba3* calculated from the data in Table 5.1 combined with published data of chromosome 1 markers.

Physiological characterization

The mutants were isolated on the basis of their resistance to paclobutrazol. Figure 5.2 shows that all *aba* mutants were at least 10 times more insensitive to paclobutrazol than the wild-types. Apparently, a lower need for GA during germination as described for the *aba1* mutant (Karssen and Laçka, 1986) results in the ability of ABA-deficient mutants to germinate in the presence of GA biosynthesis inhibitors, such as paclobutrazol.

The mutant line CB2a-9 (*aba3-2*) has been isolated by means of a screen for salt (NaCl) tolerance. This mutant and all other *aba* mutants were slightly insensitive to NaCl at germination (Fig. 5.3) as determined in a germination assay on filter paper. To investigate whether this is a general effect due to lower water potential or a specific NaCl effect, germination on a range of PEG concentrations was determined (Fig. 5.4). This indicated that the *aba* mutants were only slightly less sensitive to osmotic stress at germination than the wild-type seeds.

The induction of dormancy during development of the *aba2* and *aba3* mutant seeds was investigated (Fig. 5.5). In contrast to the wild-type, but similar to

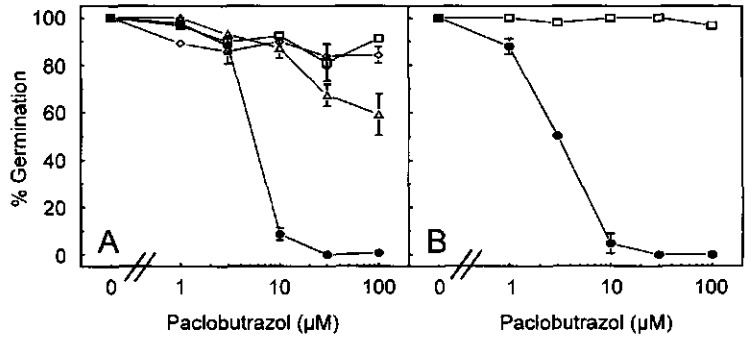

Figure 5.2. Paclobutrazol sensitivity of *aba* mutants. Germination of (A) wild-type Col (●), J11 (*aba1-5*) (◇), J12 (*aba2*) (△), and J25 (*aba3-1*) (□) seeds and of (B) wild-type L*er* (●) and CB2a-9 (*aba3-2*) (□) seeds on different concentrations of paclobutrazol.

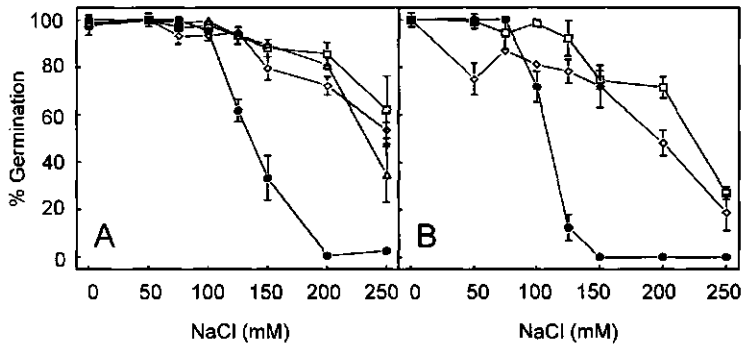

Figure 5.3. NaCl sensitivity of *aba* mutants. Germination of (A) wild-type Col (●), J11 (*aba1-5*) (◇), J12 (*aba2*) (△), and J25 (*aba3-1*) (□) seeds and of (B) wild-type L*er* (●), A26 (◇) and CB2a-9 (*aba3-2*) (□) seeds on different concentrations of NaCl.

aba1 mutants (Karssen et al., 1983), no dormancy was induced in *aba2* and *aba3*, resulting in a high germination percentage at maturity of the seeds (20 days after flowering).

The amount of ABA was measured in leaf rosettes with or without water stress (Table 5.3). Wild-type plants accumulated ABA approximately 10-fold upon water stress. The mutants *aba2* and *aba3* had lower levels of ABA in turgid condition and did not accumulate ABA upon water stress as strongly as the wild-type. Spraying the mutants with the ABA analogue LAB 173.711 restored the wild-type phenotype. The lack of adequate amounts of ABA in the vegetative parts of the plants led to a higher transpiration rate as was demonstrated by measuring the decrease in fresh weight of plants detached from their root system (Fig. 5.6). The rate of water loss was higher in the mutants than in the wild-type. The relatively low rate of water loss of mutant line J25 correlated with the relatively high ABA content in this mutant (Table 5.3).

Double mutant analysis

Lines carrying two ABA-deficient mutations were constructed. The *aba* mutants were less vigorous, with smaller rosettes and thinner flower stems than the wild-type, resulting in a lower fresh weight. In Figure 5.7 the fresh weights of rosettes just after bolting and the degree of transpiration are given, showing that the *aba1-5,aba2-1* and the *aba2-1,aba3-1* plants were more reduced in growth and showed a higher transpiration rate than the single mutants. These results indicate a slightly additive effect of the mutations, which is in agreement with the lower ABA content measured in vegetative tissues of the double mutants (Table 5.3).

To investigate the effect of the combination of ABA insensitivity and ABA-

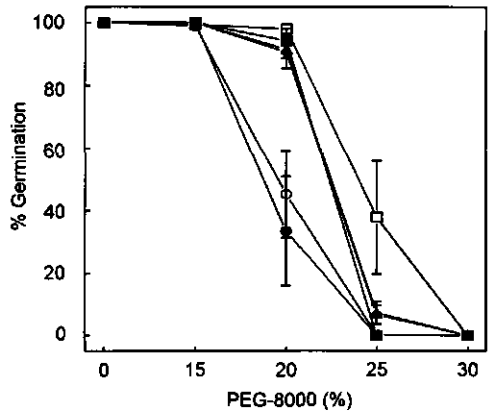

Figure 5.4. PEG sensitivity of *aba* mutants. Germination of wild-type Col (●), wild-type L*er* (○), A26 (*aba1-1*) (◊), J12 (*aba2*) (▲), J210 (*aba3*) (■), and CB2a-9 (*aba3-2*) (□) seeds on different concentrations of PEG-8000.

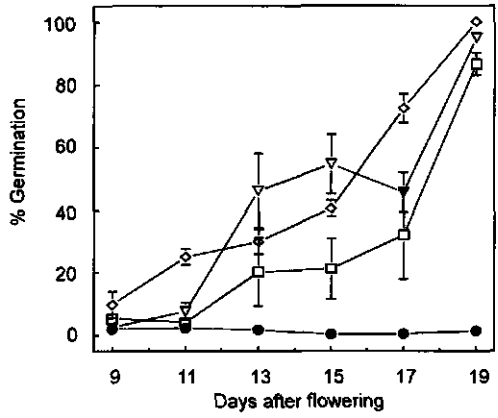

Figure 5.5. Germination on water of wild-type Col (●), J11 (*aba1-5*) (◊), J14 (*aba2-1*) (▽), and J25 (*aba3-1*) (□) seeds that were taken out from siliques at different stages during development.

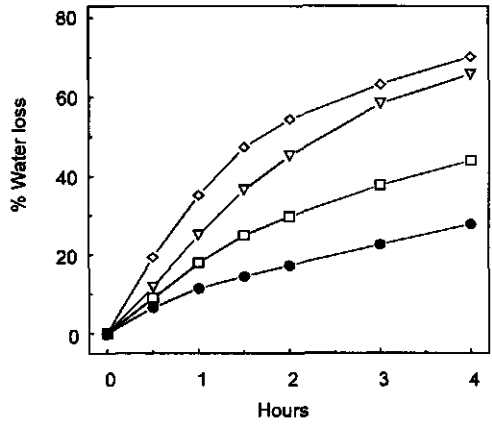

Figure 5.6. Rate of water loss of *aba* mutants. The rate of water loss of the wild-type Col (●), J11 (*aba1-5*) (◊), J14 (*aba2-1*) (▽), and J25 (*aba3-1*) (□) plants is expressed as percentage lost of initial weight in a time course of detached rosettes.

Table 5.3. Abscisic acid content of turgid and partly dehydrated rosettes. Data are averages of measurements of two samples.

Line	Genotype	Turgid	Stressed
		µg/g dry wt.	
Col	wild-type	0.22	2.92
J11	*aba1-5*	0.02	0.06
J14	*aba2-1*	0.03	0.07
J12	*aba2*	0.04	0.11
J25	*aba3-1*	0.07	0.29
F_3(J11xJ14)	*aba1-5,aba2-1*	0.01	0.01
F_3(J14xJ25)	*aba2-1,aba3-1*	0.01	0.02
Ler	wild-type	0.18	1.61
CB2a-9	*aba3-2*	0.09	0.14

deficiency of the new mutants on seeds, *aba2-1,abi3-1* and *aba3-1,abi3-1* double mutants were constructed. Seeds of the *aba3-1,abi3-1* double mutant were green, similar to the *aba1-1,abi3-1* seeds (Koornneef et al., 1989), but were not as desiccation intolerant as *aba1-1,abi3-1* seeds: after two months of dry storage of *aba3-1,abi3-1* seeds, 50% had retained the ability to germinate. Seeds of the *aba2-1,abi3-1* double mutant seeds were normally brown and desiccation tolerant. With respect to dormancy both double mutants showed synergistic effects. Figure 5.8 shows the germination of freshly harvested seeds in light and dark conditions. Seeds of both wild-types were dormant, resulting in a very low germination percentage. The single mutants were non-dormant, reflected in the high germination percentage in light but did not germinate in the dark. The high dark germination of the double mutant seeds indicated that these had a lower degree of dormancy than the seeds of the single mutants.

Discussion

ABA-deficient mutants of *Arabidopsis* have been isolated on the basis of their reduced or absent GA requirement for germination; either by screening for germinating revertants from GA-deficient mutagenized seeds (Koornneef et al., 1982) or by selection for germinating seeds on the GA inhibitor paclobutrazol (this report). A screen on a GA inhibitor has been used previously to isolate extreme *abi3* alleles (Nambara et al, 1992). GA requirement at germination is dependent on the degree of dormancy, which is in turn determined by the amount of ABA present during seed development (Karssen and Laçka, 1986). Reduced dormancy is an important characteristic of ABA-deficient mutants also in other plant species

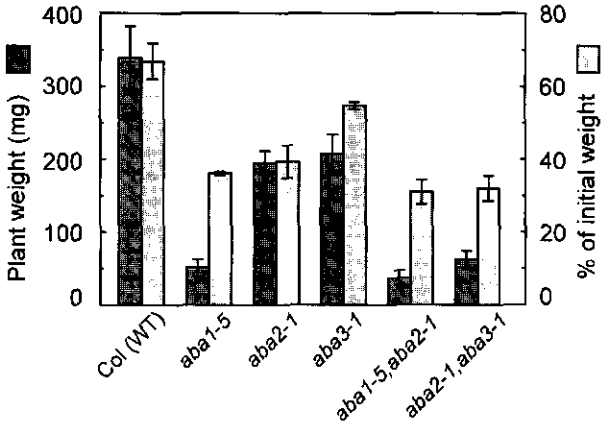

Figure 5.7. Effect of *aba* mutations on plant growth and transpiration. Plant weight after 21 days of culture and the percentage of initial weight 4.5 h after separating plants from the root system are given for *aba* mutants and double mutants. The data for the double mutants were compared with the data for their respective single mutants by t-test and were found to be significantly different ($P<0.05$).

(Koornneef, 1986).

ABA-deficient mutants of other species have been isolated by other criteria: in tomato, potato and pea on the basis of their wilty phenotype. ABA-deficient mutants of *N. plumbaginifolia* have been isolated by means of selection for cytokinin and auxin resistance. Furthermore these mutants express a reduced dormancy and resistance to paclobutrazol (Rousselin et al., 1992). All *Arabidopsis aba* mutants have the latter characteristics in common with the *N. plumbaginifolia* mutants, but are not resistant to cytokinin and auxin as was tested for *aba1* (Rousselin et al., 1992), and confirmed for *aba2* and *aba3* (data not shown).

The *aba3-2* mutant was found in a screen for salt tolerance and showed a more vigorous growth on a medium containing 200 mM NaCl than the wild-type. It is difficult to understand that a plant with lowered levels of a hormone involved in stress adaptation can do so. Nevertheless, all *aba* mutants and *abi* mutants (Werner and Finkelstein, 1995) are salt tolerant and, to a lesser extent, low osmotic potential-tolerant at germination. This is in agreement with, but less pronounced than the situation in tomato, of which the *sit* mutant is able to germinate on a medium with a much lower osmotic potential than the wild-type (Groot and Karssen, 1992; Koornneef et al, 1985; Ni and Bradford, 1993). The ability to germinate in adverse conditions such as low osmotic potential is considered to be a reflection of the reduced dormancy as well. *Arabidopsis* mutants, able to germinate on elevated NaCl concentrations have been isolated (Saleki et al., 1993; Werner and Finkelstein, 1995), but no effect on dormancy was reported. The salt resistant *rss* mutant was not wilty like *aba* mutants. The *RSS* gene is located on chromosome 1, but it is unlikely to be allelic to *aba2* because close linkage with the molecular marker *ADH* (located near *gl2*) and no linkage with *GAPB* (located near *ch1*) was reported (Werner and Finkelstein, 1995).

In the *aba* mutants analyzed here, the ABA levels correlated with the

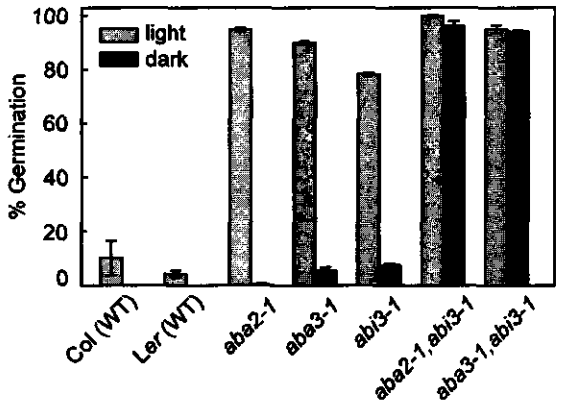

Figure 5.8. Germination of freshly harvested seeds of the wild-types, *aba,abi3-1*, and *aba,abi3-1* double mutants in light and darkness.

severity of the phenotype, expressed as wiltiness (rate of water loss) and plant weight. Plant growth in *aba* mutants is less vigourous because of their disturbed water relations. The *aba1,aba2* and *aba2,aba3* double mutants showed a more severe phenotype than the single mutants with respect to plant fresh weight, transpiration rate, and ABA levels. The presence of significant amounts of ABA in the single mutants and the lower levels of ABA in the double mutants indicate that the mutants have a leaky phenotype. Most likely, the three loci are involved in separate steps of ABA biosynthesis, assuming that the C_{40} route is the main route in *Arabidopsis* (Rock and Zeevaart, 1991). It is not clear why no mutants with a stronger reduction in ABA content were found since the presently described mutants are relatively vigorous and more extreme mutants should have been viable. The presence of relatively high ABA levels in clearly ABA-deficient mutants was also described for the *N. plumbaginifolia aba2* mutant (an orthologue of the *Arabidopsis aba1* mutants) where this was found in the null mutants used to clone the gene (Marin et al., 1996). This may suggest that some redundancy for the ABA pathway exists, which might be another explanation for the additive effect of the mutants. Furthermore it may indicate that some of the ABA produced is not physiologically active and/or that a relatively high threshold of ABA is required for processes such as stomatal closure and seed dormancy induction. It cannot be excluded that the mutations are leaky and that extreme alleles have not been recovered.

The phenotype of the *aba3-1,abi3-1* double mutant resembles that of the *aba1-1,abi3-1* double mutant (Koornneef et al., 1989): a very low degree of dormancy (expressed as high dark germination) and green seeds. Nevertheless, the *aba1-1,abi3-1* double mutant is more extreme than the *aba3-1,abi3-1* double mutant with respect to desiccation intolerance. The phenotype of the *aba2-1,abi3-1* double mutant was even less extreme based on the seed colour phenotype, although

the synergistic effect was visible in the high dark germination. An explanation for this can be that the ABA levels during seed development are affected differently in *aba2* and *aba3* mutants. The double mutant phenotype is probably not only a result of the ABA level present but can also be an indirect effect of the interaction specific for an *aba* locus with the *abi3-1* mutation.

Mutants at the new ABA-deficient loci *ABA2* and *ABA3* physiologically resemble the *aba1* mutant and can be useful in the studying of ABA action and the elucidation of the ABA biosynthetic pathway(s) in plants.

Materials and methods

Mutant isolation

The mutant selection experiment using *Arabidopsis* ecotype Columbia (Col) seeds from which the mutants were isolated has been described by Jacobsen and Olszewski (1993). From this screen, mutants that germinated in the presence of 35 mg/l paclobutrazol but whose vegetative tissues were sensitive to paclobutrazol emerged and those that were wilty were analyzed further and described in this report.

Arabidopsis ecotype Landsberg *erecta* (L*er*) seeds were mutagenized with γ-irradiation as described before (Léon-Kloosterziel et al., 1996a). M_2 seeds were sown on medium consisting of Murashige-Skoog macro- and micro-elements, 2% (w/v) sucrose, 0.7% (w/v) agar and 200 mM NaCl. Seedlings that grew more vigorously than control seedlings were transferred to soil and cultivated in the greenhouse. One of the plants selected in this way was wilty.

Genetic analysis and construction of double mutants

The mutant plants were intercrossed and crossed with the *aba1-1* (A26) mutant for complementation tests. To study the inheritance of the mutations, the mutant plants were crossed with the wild-type Col. Mutants selected fom the F_2 derived fom these crosses were crossed a second time with the wild-type. The F_2 from this second backcross yielded the mutant lines used for further characterization.

For mapping, both mutants were crossed with the multiple marker line W100 (Koornneef and Stam, 1992). Refinement of the map positions was achieved by crossing the *aba2* mutant with line W4 (containing the markers *ch1*, *ap1*, and *gl2*) and the *aba3* mutant with lines W122 and W143 (containing the markers *an*, *dis1*, *ga4*, *th1*, *tt1* and *alb1*, *an*, *dis1*, and *cer1*, respectively). An *aba3,an* recombinant was crossed with the wild-type L*er*. F_2 and F_3 populations derived from these

crosses were scored for the *aba* phenotype and the morphological markers. Recombination percentages with standard errors were determined with the computer program RECF2 (Koornneef and Stam, 1992). Map locations were determined with the computer program JOINMAP (Stam, 1993). For this purpose, the linkage data obtained in the present study were added to the data set for morphological markers used by Koornneef (1994).

Double mutants were constructed by intercrossing the mutant lines J11 (*aba1-5*), J14 (*aba2-1*) and J25 (*aba3-1*). Wilty F_2 plants derived from these crosses were testcrossed with each parent, followed by determination of F_1 plant phenotype. Double mutants were those that failed to complement both parent lines. The construction of *aba,abi3* double mutants was carried out by crossing the mutant lines J14 (*aba2-1*) and J25 (*aba3-1*) mutants with the *abi3-1* (line CIV) mutant. F_2 seeds that germinated on 10^{-5} M ABA were planted in the greenhouse; subsequently wilty plants were selected among these.

ABA determinations

ABA extractions and analysis using GC were performed as described before (Léon-Kloosterziel et al., 1996a).

Germination assays

All plants for seed production were grown in an air-conditioned greenhouse (18 - 23 °C) with additional light during winter (16-hr photoperiod, Philips HPI-T/400W); for each experiment all genotypes were harvested on the same day. Developing seeds were staged by tagging flowers at the day of anthesis. Immature or mature, bulk harvested seeds were sown (50-100 seeds) in triplicate in petri dishes on filter paper (Schleicher & Schuell no. 595) saturated with water or solutions of NaCl or paclobutrazol (ICI, Bracknell, UK). The dishes were incubated in a growth room (25°C, 16-hr photoperiod, Philips TL57) and germination (radicle emergence) scored after 7 d. The average germination percentages with standard error of the triplicates were calculated. In the case of incubation on NaCl or paclobutrazol a cold treatment (4 d at 4°C) was given prior to incubation in the growth room. For dark incubation the dishes were wrapped in aluminium foil and placed in a box.

Water loss assay

Plants were grown in soil in a climate chamber (23°C, 16-hr photoperiod (Philips TL57 and incandescent bulbs) 70-80% RH) and after 21 days of culture transferred to the laboratory and cut from the root system. Of each genotype, 5 - 15 plants (0.4 -

1.4 g fresh weight) were placed in triplicate in plastic beakers on the bench at ambient temperature, and weighed every 30 minutes.

Acknowledgements

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CHAPTER 6

Arabidopsis mutants with a reduced seed dormancy

Karen M. Léon-Kloosterziel
Gerthon A. van de Bunt
Jan A.D. Zeevaart
Maarten Koornneef

Summary
The development of seed dormancy is an aspect of seed maturation, the last stage of seed development. To isolate mutants of Arabidopsis thaliana that are affected in this process, we selected directly for the absence of dormancy among freshly harvested M_2 seeds. The screen yielded two mutants exhibiting a reduced dormancy, rdo1 and rdo2, that are specifically affected in dormancy determined by the embryo. The rdo1 and rdo2 mutants show normal levels of abscisic acid and the same sensitivity to abscisic acid, ethylene, auxin and cytokinin as the wild-type. The rdo2 mutant, but not the rdo1 mutant, has a reduced sensitivity to the gibberellin biosynthesis inhibitor tetcyclacis. Double mutant analysis suggested that the RDO1 and RDO2 genes are involved in separate pathways leading to the development of dormancy. We assume that the RDO2 gene controls a step in the induction of dormancy that is most likely induced by ABA and is expressed as an increase of the gibberellin requirement for germination.

Introduction

Many plant species produce seeds that are dormant when they are released from the plant. Seed dormancy has been defined as a temporary failure of a viable seed to germinate in conditions that favour germination when the restrictive state (i.e. dormancy) has come to an end (Simpson, 1990). Dormancy is relieved by a cold treatment of seeds allowed to imbibe and during dry storage of the seeds, often referred to as after-ripening. It can be re-induced (secondary dormancy) when the conditions do not meet all requirements for germination (Karssen, 1982). The cycling of dormancy, leading to seedling emergence in specific periods of the year, is thought to reflect the seeds' responsiveness to the environmental factors temperature, light, and nitrate (Hilhorst et al., 1996).

The small crucifer *Arabidopsis thaliana* exhibits seed dormancy, allowing the seeds in the natural situation to survive the dry summer period and germinate in autumn (Baskin and Baskin, 1972). The many environmental and genetic factors that determine seed dormancy in *Arabidopsis* have been reviewed by Koornneef and Karssen (1994). Dormancy differences between seed lots can be "measured" by comparing the germination percentage of these seed lots after a specified period of dry storage.

The conditions during seed development and the duration and conditions of dry storage after harvest influence the degree of dormancy, which is expressed in the germination behaviour of the seeds. Therefore these parameters and the test conditions have to be identical when genotypes are compared. Despite these precautions, seeds from different plants of a homozygous genotype may show larger differences than statistically expected.

The nature and variability of seed dormancy determine that this trait behaves genetically more as a quantitative trait than as a qualitative trait. However, several *Arabidopsis* mutants have been described that have a reduced dormancy. One group of these mutants is affected in the testa structure or testa colour. It is hypothesized that these mutations allow an easier penetration of the radicle through the testa because this structure is a mechanical barrier (Léon-Kloosterziel et al., 1994). In addition, a number of mutants have been described in which reduced dormancy is due to the embryo genotype. Among these are the mutants at the *ABI3* locus (Koornneef et al., 1984; Nambara et al., 1992; Ooms et al., 1993), the *LEC1* locus (Meinke, 1992; Meinke et al., 1994), and the *FUS3* locus (Bäumlein et al., 1994; Keith et al., 1994). Mutants at these loci affect many aspects of the seed maturation process, during which the seed prepares for survival by the induction of dormancy, the accumulation of storage material and the acquisition of desiccation tolerance.

Mutants deficient in the hormone abscisic acid (ABA) are among the most non-dormant mutants in *Arabidopsis* and in other plant species as well (Koornneef, 1986). ABA-deficient mutants of *Arabidopsis* were selected on the basis of their lack of a gibberellin (GA) requirement for seed germination, by the isolation of germinating revertants of non-germinating GA deficient mutants (Koornneef et al., 1982). Mutants that do not need GA for germination have also been selected on the basis of their resistance to GA biosynthesis inhibitors such as paclobutrazol (Jacobsen and Olszewski, 1993; Léon-Kloosterziel et al., 1996b) and unicazol (Nambara et al., 1991). This resulted in the isolation of additional *aba* and *abi3* mutants and of the *spy* mutant which is hypersensitive to GAs. However, since the seed maturation mutant *fus3*, in contrast to *abi3*, is not resistant to such GA inhibitors (Keith et al., 1994), a lack of seed dormancy cannot be equated with resistance to GA biosynthesis inhibitors.

A direct screen for reduced seed dormancy mutants was applied to identify mutants at other loci that play a role in this process. In this paper, we present the genetic and physiological analysis of two new mutants. Since plant hormones have been reported to be involved in seed dormancy and germination (Karssen et al., 1989), attention has been paid to the effects of hormones.

Results

Mutant isolation

After γ-rays mutagenesis, 43 M_2 seedlings were selected out of approximately 5000 M_1 plants in the reduced dormancy screen. Progeny testing showed that eight stable mutants were recovered. Some of these mutants were allelic to ABA-insensitive mutants: three mutants failed to complement *abi3* and one mutant failed to complement *abi2*. Two other mutants were affected in the pigmentation of the testa, resulting in yellow and pale brown seeds. Two mutants with wild-type brown seeds exhibited reduced dormancy. These are described in this paper.

Genetics

Allelism with *abi3* was tested because leaky *abi3* mutants are also non-dormant without strong pleiotropic effects. This led to the conclusion that both mutants were not allelic to *abi3* (data not shown). The reduced-dormancy mutants were crossed reciprocally to the wild-type and intercrossed. F_1 seeds from these crosses were dormant (Table 6.1), showing that the mutant phenotypes were caused by recessive mutations at two separate loci. These observations also indicate that the mutant

Table 6.1. Germination percentages of F_1 seeds of reciprocal crosses between the mutants, *rdo1*, *rdo2*, and the wild-type (WT). The seeds were sown 3 days (3 d) and 10 days (10 d) after harvest, and germination was scored 7 days after sowing. Percentages are means of 3-7 replicates.

Male	Female					
	rdo1		*rdo2*		WT	
	3 d	10 d	3 d	10 d	3 d	10 d
rdo1	14	97	0	36	0	51
rdo2	1	40	57	97	0	61
WT	0	n.d.[a]	0	42	0	44

[a] n.d., not determined.

phenotype, i.e. the reduced dormancy, is embryo-determined since no maternal effects are present. We designated these reduced-dormancy mutants *rdo1* and *rdo2*. F_2 plants from the cross of *rdo1* and *rdo2* with wild-type were harvested to obtain individual F_3 lines. To study the inheritance of the mutations, the germination percentage of the F_3 lines and the parents was determined by sowing them one week after harvest. The frequency distribution of the germination percentages is shown in Figure 6.1. If the frequency distribution is assumed to reflect a monogenic, recessive embryo-determined inheritance of the mutant phenotype, one-fourth of the lines should fall in the mutant class and approximately one-half of the lines should have a germination percentage slightly higher than the wild-type. To show that the distribution in Figure 6.1 represents heritable variation, the progenies of F_3 lines with the lowest and the highest germination percentages were tested for germination. In Figure 6.2 the germination percentage of the F_3 is plotted against the germination percentage of the F_4. This shows that the reduced dormancy phenotype of *rdo2* breeds true in the F_4 and that the extreme classes of the frequency distribution represent the homozygous wild-types and homozygous mutants. The F_3 data (where a peak between 20 and 50% may represent the heterozygotes) together with the F_4 data are in agreement with the segregation of a single recessive gene. Non-dormant F_3 lines from the crosses wild-type x *rdo1* and wild-type x *rdo2* were used for subsequent physiological analyses. They reproducibly showed a higher germination than the wild-type, although they were grown in different seasons. This is an additional argument for the heritability of the reduced dormancy trait.

Mutant *rdo1* plants were smaller and had shorter siliques because of fewer seeds in the siliques than the wild-type (Table 6.2). The flower buds opened

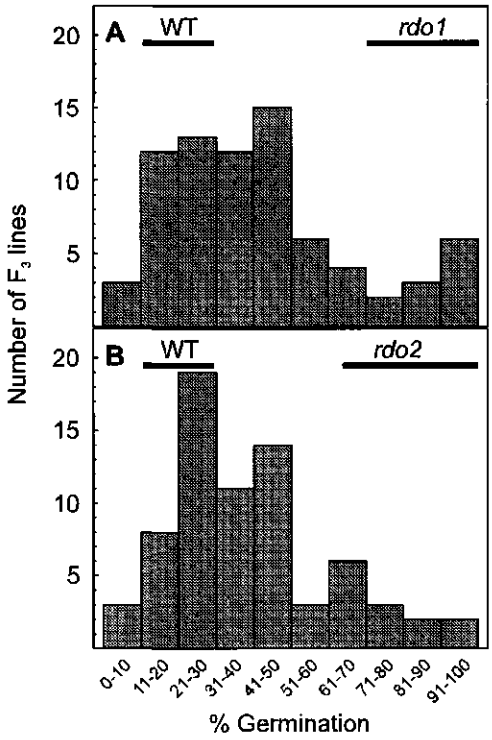

Figure 6.1. Segregation of reduced-dormancy mutants. Frequency distribution of germination percentages of individual F_3 seed batches from the crosses wild-type (WT) x *rdo1* (A) and WT x *rdo2* (B). The seeds were sown1 week after harvest and germination was scored 1 week after sowing. The bars indicate the range of germination percentages of 3 WT and 4 *rdo1* or *rdo2* lines.

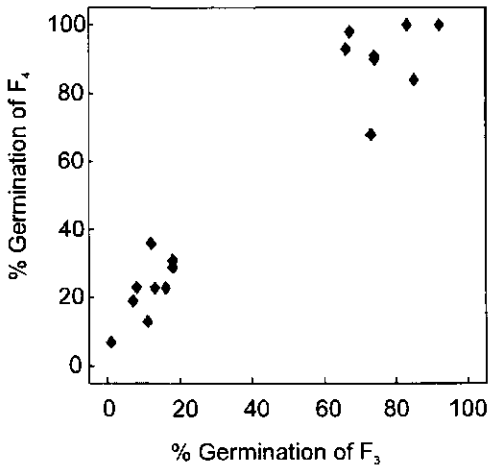

Figure 6.2. The germination percentage of a number of F_3 lines derived from the cross wild-type x *rdo2* plotted against the average germination percentage of 5 F_4 lines derived from each F_3 line. Each line represents the progeny of a single plant.

Table 6.2. Plant parameters of the wild-type (WT) and the *rdo1* mutant. The length of the main stem after termination of flowering was determined, together with the length of the sixth silique and the number of seeds, embryo-lethals, and non-fertilized ovules in this silique. Values are means of 15 determinations ± SE.

	Length of main stem	Silique length	Sum of N° of seeds, embryo-lethals, and ovules	Fertility
	cm	mm		%
WT	26.5 ± 5.3	12.2 ± 0.3	56 ± 2	95 ± 2
rdo1	17.3 ± 4.3	10.6 ± 0.4	51 ± 2	94 ± 2

prematurely. The reduced dormancy and the aberrant plant phenotype co-segregated in 140 F_3 and F_4 lines derived from the cross wild-type x *rdo1*, indicating that these are pleiotropic effects of the *rdo1* mutation and that the reduced dormancy of this mutant is a heritable trait. The phenotype of *rdo2* plants was normal except for a slightly darker green rosette than the wild-type and a slight retardation of flowering (1 to 2 days), with a normal leaf number.

Seed dormancy characteristics of the *rdo1* and *rdo2* mutants

The ability to germinate as freshly harvested seeds indicates a lack of an after-ripening requirement that is induced during seed development (Karssen et al., 1983). To follow the course dormancy induction, germination of excised developing seeds in water was determined using the wild-type as a dormant and the *aba1-1* mutant as a non-dormant control (Fig. 6.3). Wild-type seeds older than 13

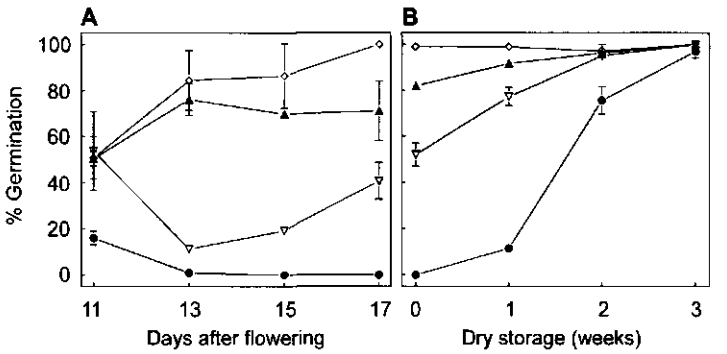

Figure 6.3. Induction and loss of dormancy. Germination in water of *rdo1* (▽), *rdo2* (▲), *aba1-1* (◊), and wild-type (●) seeds that were removed from the siliques at different stages during development (A) and of seed batches sown after different periods of storage of the seeds (B). Percentages are means of triplicates ± SE.

Table 6.3. Dark germination of reduced-dormancy mutants. Final germination percentages reached after 16 days (5°C), 5 days (15°C), and 3 days (25, 30°C) of imbibition in darkness of the wild-type (WT), dormancy mutants (*rdo1*, *rdo2*) and ABA-deficient mutant (*aba1-1*) at different temperatures are given. Percentages are means of numbers indicated (n) ± SE.

Temperature	WT	*rdo1*	*rdo2*	*aba1-1*	n
5°C	71 ± 3	89 ± 1	100 ± 0	98 ± 1	12
15°C	50 ± 3	86 ± 1	100 ± 0	99 ± 0	21
25°C	64 ± 4	98 ± 1	95 ± 1	99 ± 1	21
30°C	3 ± 1	57 ± 2	27 ± 2	87 ± 1	18

days after flowering did not germinate, reflecting their dormant state. *rdo1* seeds were slowly released from dormancy from 13 days after flowering onward (Fig. 6.3A) and this release continued during dry storage of the seeds (Fig. 6.3B). Dormancy was not induced in *rdo2* seeds. Seeds of the ABA-deficient mutant (*aba1-1*) could germinate at all stages of development. Wild-type seeds were released from dormancy during several weeks of dry storage (Fig. 6.3B). The degree of dormancy varied between seed batches of different harvest dates, reflected in differences in levels and slopes of curves such as in Figure 6.3B. The relative germination percentages were always *aba1-1* > *rdo2* > *rdo1* > wild-type.

The degree of dormancy can also be described by the ability to germinate in unfavourable conditions, such as darkness and suboptimal or supraoptimal temperatures. Table 6.3 shows that *rdo1* and *rdo2* are very similar to *aba1-1* in having a higher capacity to germinate in darkness. At 30°C, germination percentages of *rdo1* and *rdo2* decreased to lower values than those of *aba1-1*, but not as low as those of the wild-type. This confirms that the new mutants have a reduced degree of dormancy compared to the wild-type but are not as non-dormant as the *aba1-1* mutant.

Hormone levels and sensitivity

Since ABA-related mutants have a reduced dormancy, we investigated whether *rdo1* and *rdo2* were ABA-insensitive or ABA-deficient. Figure 6.4 shows that ABA-sensitivity of germination of *rdo1* and *rdo2* was slightly lower than the wild-type. Mutant plants were not wilty, suggesting that they are not ABA-deficient. ABA determinations confirmed that the *rdo1* and *rdo2* mutants had near wild-type levels of endogenous ABA (Table 6.4). Seeds of both mutants contained at least as much ABA as wild-type seeds, indicating that reduced dormancy of the mutants is not due to reduced ABA specifically in the seeds.

Ethylene can replace GA for *Arabidopsis* seed germination (Karssen et al.,

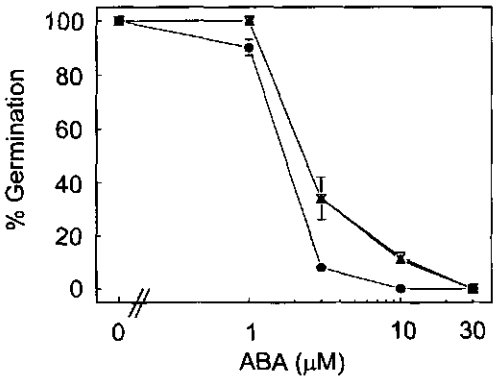

Figure 6.4. ABA-sensitivity of reduced-dormancy mutants. Germination of *rdo1* (▽), *rdo2* (▲), and wild-type (●) seeds in different ABA concentrations. Percentages are means of triplicates ± SE.

1989). Ethylene insensitive mutants of *Arabidopsis* show poor germination in relation to the wild-type (Bleecker et al., 1988). "Easily" germinating seeds, like those of *rdo1* and *rdo2* might be hypersensitive to ethylene. We investigated the ethylene sensitivity by germinating seeds in the dark in the presence of GA_{4+7} and ACC. Hypocotyl length was inhibited to the same extent in wild-type, *rdo1*, and *rdo2* seedlings (Table 6.5); therefore, *rdo1* and *rdo2* can not be classified as ethylene response mutants.

The sensitivity to inhibitory effects of the auxin NAA and the cytokinin BA was tested with a root growth assay. This did not reveal a different sensitivity of the mutants compared to the wild-type, except for a slight insensitivity with respect to root growth of *rdo1* to NAA (Table 6.5).

Since GAs are required for seed germination (Karssen et al., 1989) we determined the sensitivity to the GA biosynthesis inhibitor tetcyclacis (Figure 6.5). Sensitivity to tetcyclacis is reduced in *rdo2*, but the curve of *rdo1* corresponds with the wild-type. The differences between seed batches of different harvest dates with respect to tetcyclacis sensitivity are also shown. However, a similar relative

Table 6.4. Endogenous ABA content of turgid and partly dehydrated rosettes and seeds. Amounts in rosettes are the averages of measurements of two samples. Amounts of ABA in seeds are measurements of one sample for each genotype.

Genotype	Turgid rosettes	Stressed rosettes	Mature dry seeds
		μg/g dry weight	
Ler (WT)	0.10	1.11	0.14
rdo1	0.12	0.85	0.15
rdo2	0.12	0.89	0.36

Table 6.5. Sensitivity to ethylene, NAA, and BA. The inhibition of hypocotyl elongation on 300 µM ACC[a], and root growth on 10^{-7} M NAA or BA[a] is expressed as percentage of the control hypocotyl and roots ± standard deviations.

Genotype	ACC	NAA	BA
L*er* (WT)	38 ± 8	43 ± 7	35 ± 7
rdo1	42 ± 8	66 ± 13	41 ± 10
rdo2	39 ± 8	40 ± 9	35 ± 11

[a] Only the value of one concentration is given, which was representative for all concentrations tested.

insensitivity of *rdo2*, but not of *rdo1*, was found in four independent experiments using different seed harvest batches. The tetcyclacis sensitivity of a given seed batch does not change with time (data not shown); therefore, the differences in tetcyclacis sensitivity are due to different growing conditions and not to the storage duration of the seeds.

The tetcyclacis insensitivity was a true pleiotropic effect of the *rdo2* mutation as was tested with F_4 lines derived from the cross wild-type x *rdo2*. Figure 6.6 shows that all lines with a low degree of dormancy, expressed as a high germination percentage germinate on 100 µM tetcyclacis, whereas lines with a low germination percentage hardly germinate in the presence of tetcyclacis.

Interaction with other seed dormancy mutants

Double mutants were constructed between *rdo1*, *rdo2*, and the non-dormant mutants, *aba1-1*, *abi2-1*, *abi3-1*, and *abi3-5*. The germination rates of the different mutants and their double mutants were analyzed. The germination rates of the ABA-related mutants *aba1-1*, *abi2-1*, and *abi3-5* were higher than those of *rdo1* and *rdo2*, but the double mutants' germination rates were not at all or only slightly

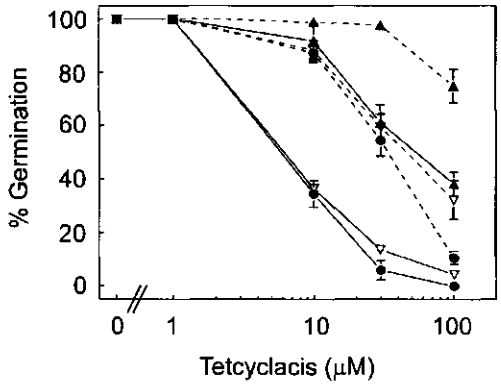

Figure 6.5. Tetcyclacis sensitivity of reduced-dormancy mutants. Seeds of *rdo1* (▽), *rdo2* (▲), and the wild-type (●) harvested in June, 1994 (solid lines) and October, 1994 (dotted lines) were tested for their ability to germinate on different concentrations of tetcyclacis. Percentages are means of triplicates ± SE.

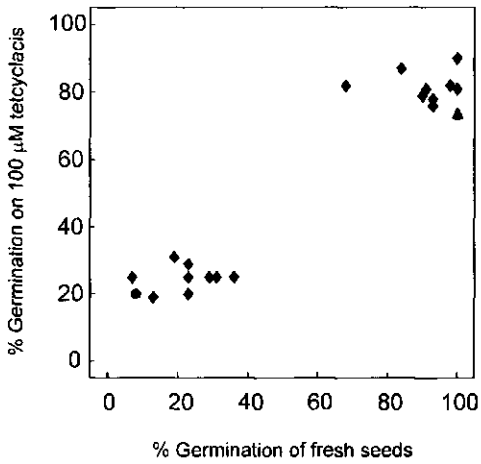

Figure 6.6. The correlation between reduced dormancy and tetcyclacis insensitivity. The germination percentage of freshly harvested seeds of F_4 lines derived from the cross wild-type x *rdo2* (◆), the wild-type (●), and the *rdo2* parent (▲) is plotted against the germination percentage on 100 µM tetcyclacis.

higher than those of the single mutants (not shown). The germination rate of the *rdo1,rdo2* double mutant is given in Figure 6.7A. The germination rate of the *rdo1,abi3-1* double mutant was higher than the sum of the single mutants' germination rates indicating an amplifying effect of these two mutations (Fig. 6.7B). In contrast to this, the *rdo2,abi3-1* germination rate was not higher than the sum of the germination rates of *rdo2* and *abi3-1* (Fig. 6.7C). This additive effect of *rdo1* to *abi3* was also visible in the loss of viability of *abi3* seeds. The extreme mutant alleles *abi3-4* and *abi3-5* have a reduced desiccation tolerance (Ooms et al., 1993). The time after which one-half of the seeds have lost their viability was 2 times shorter for *rdo1,abi3-5* mutant seeds than for *abi3-5* and *rdo2,abi3-5* seeds.

Discussion

In this paper, two *Arabidopsis* mutants that are specifically affected in the degree of dormancy are described. They originated from a screen for mutant seeds that were able to germinate immediately after harvest. Such a screen can be expected to yield ABA-related mutants since these lack dormancy. The ABA-insensitive mutants *abi2* and *abi3* were found, but no additional ABA-deficient mutants emerged. Two mutants were seed colour mutants. A reduced dormancy is common for seed colour mutants such as the *ttg* and *tt* (*transparent testa*) mutants (Koornneef, 1981; Léon-Kloosterziel et al., 1994; Debeaujon, 1995); therefore, it was concluded that the reduced dormancy of the colour mutants found in our screen was most likely caused by a mutation affecting the testa pigmentation. However, since the testa is maternally inherited and *tt* mutants are recessive, it is not likely that the mutant testa phenotype was present in the M_2 generation in which selection was applied. A

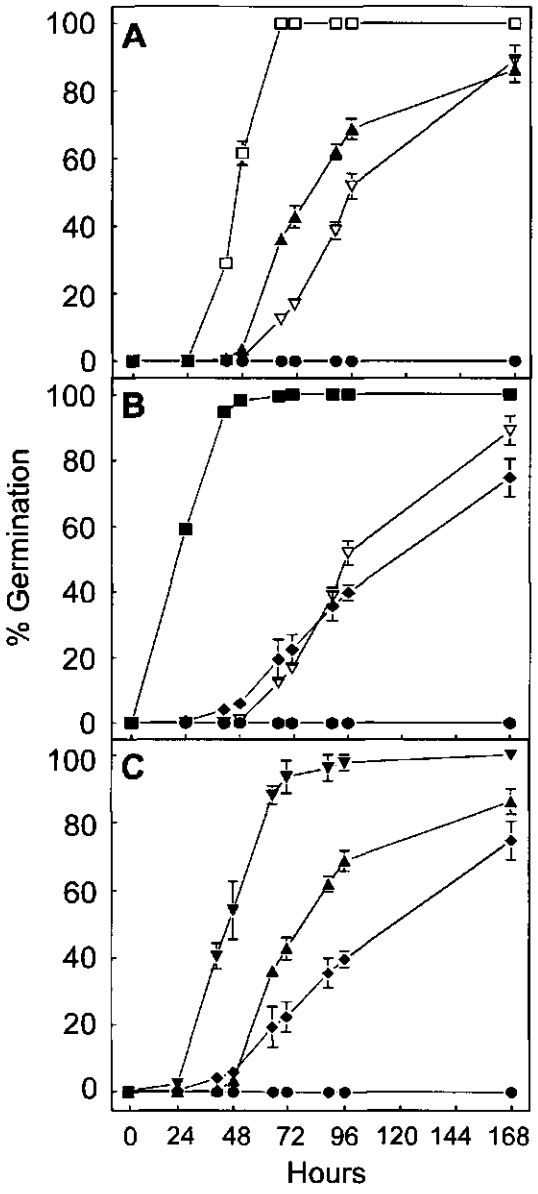

Figure 6.7. Germination rate of freshly harvested seeds. A, Germination rates of the wild-type (●), the *rdo1* (▽) and *rdo2* (▲) mutants and the *rdo1,rdo2* double mutant (□). B, Germination rates of the wild-type L*er* (●), the *rdo1* (▽) and *abi3-1* (◆) mutants and the *rdo1,abi3-1* (■) double mutant. C, Germination rates of the wild-type (●), the *rdo2* (▲) and *abi3-1* (◆) mutants and the *rdo2,abi3-1* double mutant (▼). Percentages are means of triplicates ± SE.

slight dominance, not detectable on the basis of seed colour, might influence dormancy and explain the finding of these mutants in the M_2. However, it can not be excluded that originally these mutants have been "false positives", which turned out to have a seed colour mutation.

The degree of dormancy of a seed lot is reflected in the germination percentage relatively soon after harvest of the seeds, since the release from dormancy of *Arabidopsis* ecotype Landsberg *erecta* (L*er*) occurs within several weeks of dry storage of the seeds (unpublished observations). *Arabidopsis* is known to show a large variability in light and GA requirement for seed germination, due to differences in growing conditions (Derkx and Karssen, 1993b). We found that the degree of dormancy and coherently the rate of release from dormancy of the mutants was always higher than that of the wild-type but highly variable between seed batches of different harvest dates. To perform a physiological analysis of these mutants, comparisons had to be made between seed lots grown at the same time and in the same conditions. We also repeated most experiments three times to ascertain consistent results. An example of the variability with respect to tetcyclacis sensitivity is shown in Figure 6.5.

The reduced dormancy trait of the mutants is caused by a single recessive mutation at two distinct loci, *rdo1* and *rdo2*, and is embryo-determined (Fig. 6.1, Table 6.1). Dormancy can vary between ecotypes and can be influenced by several mutations present in marker lines. For this reason we did not attempt to locate these mutations on the genetic map of *Arabidopsis*. The difficulty in performing genetics on variable traits like reduced dormancy is also illustrated in Figure 6.1 where no clear phenotypic classes can be distinguished.

Lack of dormancy can have a hormonal cause as described for ABA-deficient (*aba*) or ABA-insensitive (*abi*) mutants (Koornneef et al., 1982, Koornneef et al., 1984). The *rdo1* and *rdo2* mutants cannot be classified as ABA-related mutants since their ABA content was normal (Table 6.4) and *rdo1* and *rdo2* seeds were not ABA-insensitive at germination (Fig. 6.4). The curves of *rdo1* and *rdo2* differed slightly from that of the wild-type, but this does not indicate significant ABA-insensitivity since ABA-insensitive mutants are at least 10 times more insensitive to ABA at germination than the wild-type (Koornneef et al., 1984; Finkelstein, 1994). The possibility that the reduced dormancy originates in an altered sensitivity to ethylene, auxins, or cytokinins was ruled out by the observation that the mutants and the wild-type exhibited the same hypocotyl inhibition by the ethylene precursor ACC and the same root growth inhibition by NAA and BA.

The mutants *rdo1* and *rdo2* are not ABA-deficient in either vegetative parts or in mature seeds. In mature seeds, embryonic ABA, known to be responsible for dormancy induction, prevails (Karssen et al., 1983). Apparently, the *rdo* mutants do not represent the class of maize mutants that are specifically ABA-deficient in the seeds (McCarty, 1995). However, the *rdo* mutants have physiological characteristics similar to those of the *aba1* mutant. Similar to the *aba1* mutant, the reduced dormancy of *rdo1* and *rdo2* result in a broader germination window (Table 6.3). Furthermore, seeds of the *aba1* and *rdo2* mutants, but not the *rdo1* mutant, were more resistant to the GA biosynthesis inhibitor tetcyclacis (Fig. 6.5). This indicates that less GA is required for germination than in the wild-type. Since the plant height phenotype of *rdo2* was normal, it is not likely that the GA response is altered in this mutant. The *spy* mutant of *Arabidopsis*, which is non-dormant and hypersensitive to GA shows a phenotype comparable with GA-treated plants (Jacobsen and Olszewski, 1993).

The *rdo2* mutants have a lower GA requirement specific for seed germination in common with ABA-deficient (Koornneef et al., 1982; Léon-Kloosterziel et al., 1996b) and ABA-insensitive mutants (Nambara et al., 1991). However, a lack of ABA or ABA action does not seem to be the cause of this lack of dormancy accompanied by a reduced GA requirement for germination. It is likely that the *RDO2* locus controls a step between the initial occurrence of ABA and ABA induced dormancy. Apparently, this step is specific only for the ABA effects on seed dormancy since no pleiotropic effects on water relations, like those observed in *aba* and *abi* mutants, are present in *rdo2*.

A reduced seed dormancy is not always associated with a reduced GA requirement for germination, since the *rdo1* mutant is as sensitive to GA biosynthesis inhibitors as the wild-type. The same was reported for the *fus3* mutant, which is extremely non-dormant and is strongly disturbed in seed maturation (Bäumlein et al., 1994; Keith et al., 1994). Double mutant analysis led to the conclusion that *FUS3* and *ABI3* control parallel pathways that regulate seed maturation. The reduced dormancy in the *fus3* mutant does not abolish the GA requirement for germination and *RDO1* may control a step in the same dormancy mechanism as the one that is affected in the *fus3* mutant.

This can be explained by posing the hypothesis that dormancy has two aspects controlled by different mechanisms in the embryo tissue. Dormancy is induced in a pathway in which the *FUS3* and *RDO1* genes are involved. This is one aspect of the seed maturation process and is leading to a developmental arrest. A separate pathway, in which the *ABA1*, *ABI3*, and *RDO2* genes are involved, induces a requirement for *de novo* GA biosynthesis, in addition to this developmental arrest.

This model predicts that the *rdo1,rdo2* and the *rdo1,abi3* double mutants

show an additive effect and that the *rdo2,abi3* double mutant phenotype is like the single mutant's phenotypes, except when the mutants are leaky. The *abi3-1* allele is leaky because most other alleles described have a much more extreme phenotype (Koornneef et al., 1984; Nambara et al., 1992; Ooms et al., 1993). Since we are not certain that the *rdo1* and *rdo2* alleles we isolated are null alleles, the double mutant analysis may not be completely conclusive. However, the *rdo1,rdo2* and the *rdo2,abi3-1* double mutants showed an additive germination rate and the *rdo1,abi3-1* germination rate was higher than the *rdo2,abi3-1* germination rate, confirming the model of parallel pathways (Fig. 6.5). Desiccation tolerance, which was low in *abi3-5* mutants is even more reduced in *rdo1,abi3-5* double mutants. This also confirms the hypothesis that *RDO1* and *ABI3* act in different pathways and may mean that although the *rdo1* mutant is desiccation tolerant, the *RDO1* gene is involved in this other aspect of seed maturation as is the *FUS3* gene.

Materials and methods

Mutant isolation

M_1 Seeds of *Arabidopsis thaliana* (L.) Heyhn, ecotype Landsberg *erecta* (L*er*) were imbibed in tubes containing 0.2% agar for 20 hr at 4°C, mutagenized with 200 or 300 Gy γ-irradiation (^{60}Co source), and dispersed on soil in an air-conditioned greenhouse (18-23°C) with additional light during winter (16 hr photoperiod, Philips HP1-T/400 W). M_2-seeds were harvested from groups of approximately 500 M_1 plants and sown on water saturated filter paper (Schleicher and Schuell N° 595) in petri dishes on the day of harvest. The dishes were incubated in a growth room (25°C, 16 hr photoperiod, Philips TL57). Seeds that had germinated within 3 days after imbibition were selected as putative mutants. Freshly harvested wild-type seeds did not germinate under these conditions (less than 1%). The mutant phenotype was confirmed by re-testing the germination of the seeds harvested from plants grown from these selected seedlings.

Genetic analysis

The mutant plants were crossed with the wild-type. Non-dormant F_3 lines derived from this cross were used for reciprocal crosses and physiological analyses. The non-dormant mutants were also crossed among themselves and with *abi2-1*, *abi3-1* (Koornneef et al., 1984), *abi3-5* (Ooms et al., 1993), and *aba1-1* (Koornneef et al., 1982) mutants to perform complementation tests and to construct double mutants. Double mutants were selected on the basis of ABA-insensitivity (*abi2-1*, *abi3-1*),

green seeds (*abi3-5*), and wilty plants (*aba1-1*). Double mutants were selected by reciprocal test crossing with each of the non-dormant parents followed by determination of the F_1 seed germination.

Germination assays

All genotypes were grown together and mature seeds were harvested at the same time from dehydrated siliques. Batches of 50 to 80 seeds were sown in triplicate in petri dishes containing water soaked filter paper (Schleicher and Schuell n° 595) and incubated in a growth room (25 °C, 16 hr photoperiod). Seeds sown on solutions of ABA (Sigma) or tetcyclacis in water were incubated for 3 d at 4 °C prior to transfer to the growth room. Germination was scored after 7 d of incubation at 25 °C.

Determination of sensitivity to ethylene, auxin, and cytokinin

Sensitivity to ethylene was determined by sowing seeds on 0, 30, 100, and 300 µM 1-aminocyclopropane-1-carboxylic acid (ACC, Sigma) and 10^{-4} M gibberellin (GA_{4+7}, ICI, Bracknell, UK) on filter paper in sealed petri dishes. Both a mutant and the wild-type were sown in each dish and incubated in darkness at 22°C after 1 d of cold treatment (4°C). Hypocotyl length was determined after 6 d incubation at 22°C. Sensitivity to the auxin 1-naphtaleneacetic acid (NAA, Sigma) and to the cytokinin benzyladenine (BA, Sigma) was determined by a root growth assay in vertical petri dishes. Seedlings were grown in a vertical position on 4.59 g/l Murashige and Skoog minerals and micronutrients, 2% (w/v) sucrose and 0.7% (w/v) agar with 10^{-9}, 10^{-8}, 10^{-7}, or 10^{-5} M NAA or BA. Root length was determined after 10 d of incubation in a growth room (25°C, 16 hr photoperiod), after 3 d of cold treatment (4°C).

ABA determinations

Plants were grown under the same conditions as previously described (Rock and Zeevaart, 1991) and used for ABA determinations when still in the rosette stage. In all experiments, ABA content of turgid and water-stressed plants was compared. Turgid material was harvested and frozen immediately in liquid N_2. In the case of water stress, detached rosettes were dehydrated in dry air at room temperature until 15% of the fresh weight was lost. The stressed material was kept in polyethylene bags at 22°C for 5 to 6 hr and then frozen in liquid N_2. The frozen material was lyophilized and the dry weight was determined.

The lyophilized material was extracted as described before (Cornish and Zeevaart, 1984). To each extract was added 20,000 dpm of [^{3}H]ABA to quantify losses during purification. The dried crude extracts were first purified by

semipreparative reverse phase HPLC with a μBondapak C_{18} column (30x0.78 cm) (Rock and Zeevaart, 1991). The fractions containing ABA were collected, dried, and methylated with ethereal diazomethane. Me-ABA in these fractions was further purified by normal phase HPLC with a μPorasil column (30x0.4 cm) (Zeevaart et al., 1989). Quantification of Me-ABA was performed by GLC with electron capture detector (Cornish and Zeevaart, 1984).

Dry seeds (approximately 1 g of wild-type and of each mutant, from seed batches grown and harvested at the same time and stored for 14 months) were extracted overnight. The samples were homogenized with a Polytron (Brinkmann) and the extracts were filtered. The acetone was evaporated, and the oil extracted from the seeds was removed by partitioning the aqueous concentrate with hexane. The water phase was frozen and lyophilized. ABA in the residue was purified by HPLC and measured as described above for plant material.

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CHAPTER 7

General Discussion

General Discussion

Mutant screens

The investigation of physiological processes in higher plants by means of mutant analyses has appeared to be successful in *Arabidopsis*. The work described in this thesis is a contribution to the genetic dissection of seed development. Different mutant screens have been applied and have resulted in the isolation of additional members of previously described classes and also of new classes of seed maturation mutants. The mutant screens are summarized in Table 7.1. Mutant selections, based on deviating morphological and physiological phenotypes, have been performed in material mutagenized with chemicals, γ-irradiation, T-DNA, and transposons. In addition, preliminary experiments were performed to exploit the natural variation for seed traits, present among *Arabidopsis* ecotypes. Such genetic differences between ecotypes for seed dormancy for example have been reported before (reviewed in Koornneef and Karssen, 1994).

Several screens resulted in the isolation of abscisic acid (ABA)-related mutants. The screen for germination on high ABA concentration and for green seed phenotypes yielded additional *abi3* mutants. Alleles of *abi3* were also isolated by a direct screen for reduced dormancy, a characteristic of both ABA-deficient and -insensitive mutants. The germination of *Arabidopsis* seeds requires gibberellins (GA). This is shown by the absence of germination in GA-deficient mutants (Koornneef and Van der Veen, 1980) and the inhibition of germination by inhibitors of GA biosynthesis (Karssen et al., 1989). The absence of dormancy abolishes the GA requirement for germination as was indicated by the isolation of ABA-deficient mutants and of mutants resistant to GA biosynthesis inhibitors such as uniconazol and paclobutrazol (Koornneef et al., 1982; Nambara et al., 1992; Jacobsen and Olszewski, 1993). In accordance with this strategy, additional ABA biosynthesis mutants were isolated among the paclobutrazol insensitive mutants of Jacobsen and Olszewski (1993). In the reduced-dormancy mutant *rdo2* paclobutrazol resistance was also observed (Léon-Kloosterziel et al., 1996a). Furthermore, it appeared that ecotypes can differ in their resistance to GA biosynthesis inhibitors. For instance, the ecotype Col is 2-3 fold more resistant to paclobutrazol than *Ler* (Van der Schaar et al., 1997). However, the finding that the *rdo1* mutant is not paclobutrazol resistant indicates that this resistance is not a common characteristic of all reduced dormancy mutants. The fact that genes that control paclobutrazol resistance are located at positions different from dormancy genes also suggests that

Table 7.1. Mutant screens performed to isolate seed maturation mutants.

Screen	Mutagens	Background	Mutant(s)	Chapter
Reduced dormancy	EMS, γ T-DNA	L*er*, *abi3-1* (L*er*) Ws	*rdo1*, *rdo2*, *abi2*, *abi3* *aba1*, *ap2*	6 -
Reduced secondary dormancy	T-DNA	Ws	*tt12*	-
Germination on GA biosynthesis inhibitor (paclobutrazol)	EMS	Col	*aba1*, *aba2*, *aba3*	5
Germination and growth on medium with high NaCl	γ	L*er*	*aba3*	5
Germination on 100 µM ABA	EMS	*abi3-1* (L*er*)	*abi3-11*	4
Non-germinating seeds	EMS, γ	*abi3-1*, *aba1* (L*er*)	GA-deficient	-
Green seeds	EMS, DEB	L*er*, *abi3-1* (L*er*)	*abi3-4*, *abi3-5*	3
Anthocyanin accumulation in immature seeds	transposon (En/I)	L*er*	*lec1-3*	4
Extreme seed sizes	γ	L*er*	-	-
Reduced/enhanced EmGUS expression	γ	L*er*/C24	reduced-Em mutants	-

paclobutrazol resistance is not always a consequence of lack of dormancy (Van der Schaar et al., 1997).

Many mutant screens that have been described in literature and that have yielded seed-specific mutants were based on a morphological phenotype such as the presence of trichomes on the cotyledons or purple seeds. This led to the identification of the three genes *LEC1*, *LEC2* and *FUS3* that, together with *ABI3*, seem to be crucial regulating genes for seed maturation (Meinke, 1992; Bäumlein et al., 1994; Keith et al., 1994; Meinke et al., 1994; West et al., 1994). Further screens were specifically based on the resistance/sensitivity to ABA. This has resulted in the isolation of the *abi4* and *abi5* mutants that are slightly ABA-insensitive (3-10 fold more insensitive than the wild-type) and are similar to the weak *abi3* alleles in terms of phenotype and interactions with the other *abi* mutants (Finkelstein, 1994). A screen for enhanced ABA-sensitivity at germination described by Cutler et al. (1996) resulted in the isolation of the *era1* (*enhanced response to ABA*) mutant. The enhanced ABA-sensitivity is also reflected in an elevated level of dormancy in mutant seeds.

A different kind of screen to obtain mutants that are specifically affected in seed maturation is by selecting for altered gene expression in plants containing a specific promoter driving the expression of a reporter gene. After mutagenesis, screens for the absence or constitutive presence of expression of the reporter gene can lead to the isolation of mutants in signal transduction that may not have an obvious phenotype on their own. This approach has been used for example for the isolation of a mutant that was non-responsive to inducers of systemic acquired resistance (Cao et al., 1994). In a collaboration between our laboratory and the laboratory of Dr. Delseny at the University of Perpignan (France) this approach has also been applied to isolate seed maturation mutants. For this, the promoter of the AtEm1 gene, which is an ABA inducible LEA gene only expressed in seeds (Gaubier et al., 1993), was fused to the GUS-reporter gene and introduced into *Arabidopsis* (Hull et al., 1996). This transgenic line was mutagenized with γ-radiation and seedlings with reduced and enhanced GUS-expression were selected, using a non-destructive GUS-assay. Some mutants with a reduced GUS-expression were shown to have a lower Em mRNA and Em protein accumulation. These mutants were not ABA- or paclobutrazol-insensitive. Although the Em protein is often suggested to be involved in the induction of desiccation tolerance, these expression mutants were normally desiccation tolerant and showed normal longevity (Bies, 1997).

The accumulation of storage lipids and proteins is a characteristic of seed

maturation. Therefore it can be expected that disturbed regulation of these processes could lead to differences in seed morphology or size. The selection for small and shrivelled (but germinable) seeds led to the isolation of the *wri* mutants (Focks and Benning, 1996). Selection for larger seeds was performed by means of sieves with appropriate pore sizes. Two mutants with consistently larger seeds could be selected, but were not characterized further. Genetic differences for seed size are also present among ecotypes (Krannitz et al., 1991). Presently, the large seed size character of the Cvi ecotype is genetically analyzed using recombinant inbred lines (C. Alonso-Blanco, personal communication).

Seed maturation

Regulation of seed maturation is a complex network in which many genes are involved. The genes identified by their mutant phenotype can be integrated in a seed maturation model as schematically represented in Figure 7.1. Characteristics of the mutants are summarized in Table 7.2. *LEC1*, *LEC2* and *FUS3* can be considered as general regulatory genes of the seed maturation process. They have a function only in this process since no phenotypic effects are observed at the seedling (except for the presence of trichomes on the cotyledons) and adult plant stage. These genes might control, together with *ABI3*, the expression of seed maturation-specific target genes, such as those affecting the accumulation of storage lipids and proteins, LEA proteins, but also other, still unknown genes that control desiccation tolerance and seed dormancy. The *ABI3* gene is required for the ABA action in seeds and thereby links these processes with ABA biosynthesis and the early steps of ABA signal transduction. The *abi3* mutation inhibits the accumulation of multiple seed maturation-specific transcripts, but does not globally disrupt all programs of seed maturation gene expression (Parcy et al., 1994). Gene expression studies in the *abi3*, *lec* and *fus* mutants have shown that separate sets of transcripts, that partly overlap, are not induced (Bäumlein et al., 1994; Parcy et al., 1994; West et al., 1994). In the *aba*, *abi1* and *abi2* mutants mainly seed dormancy is affected. The strong effect of *aba* mutations on dormancy suggest that the effects of ABA in seeds might be rather exclusive for dormancy development and dormancy maintenance. In contrast, expression of e.g. the Em gene is much less reduced in these mutants than in *abi3* mutants (Finkelstein, 1993; Parcy and Giraudat, 1997). This suggests that *ABI3* has a role in controlling seed maturation which is not only confined to ABA signalling. Parcy and Giraudat (1997) provided a model in which *ABI3* is required to mediate the effects of ABA in seed maturation but that developmental signals may also mediate *ABI3* action in an ABA independent way.

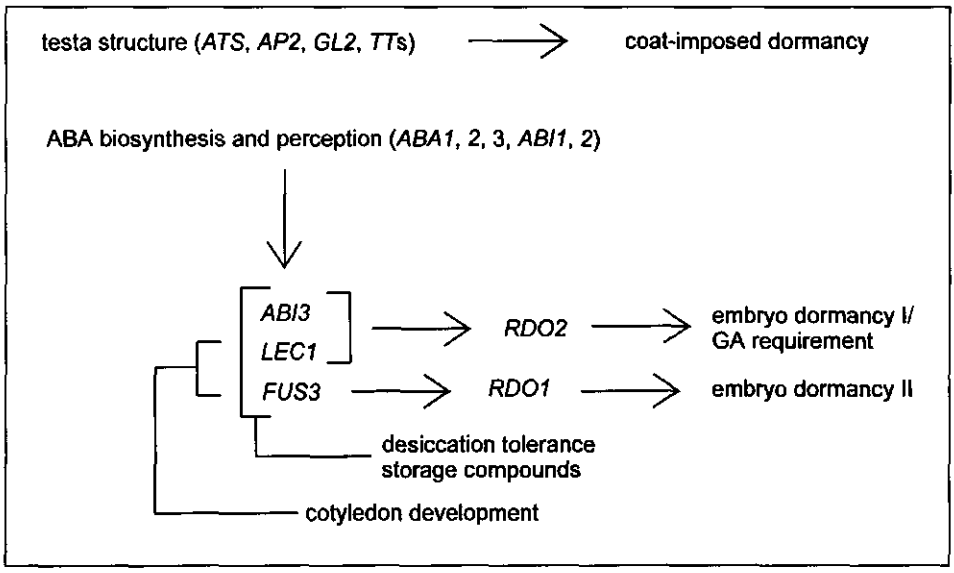

Figure 7.1. Model incorporating the various seed maturation genes identified by mutation.

Also the *LEC1* and *FUS3* genes are likely to act not independently from *ABI3*, but in the same network (Parcy et al., 1997). The *RDO* genes have an effect only on dormancy and not on other aspects of seed maturation. Therefore, they seem to be active late in the regulation after a branching into the various aspects has taken place.

Although a large number of seed maturation-specific genes have been cloned (reviewed by Delseny et al., 1994) only a limited number of the genes presented in Figure 7.1 have been cloned to date. These are the *ABA1*, *ABI1*, *ABI2*, and *ABI3* genes. The ABI1 and ABI2 proteins are suggested to be protein serine/threonine phosphatases 2C, having partially redundant functions in ABA signalling (Leung et al., 1994; Leung et al., 1997). The seed-specific *ABI3* gene appeared to be homologous to the maize *VP1* gene and encodes a transcription factor (Giraudat et al., 1992). The *ERA1* gene was also cloned and encodes a protein farnesyl transferase, suggesting that a protein that normally negatively regulates ABA signalling in seeds, may require farnesylation in order to function (Cutler et al., 1996).

Several of the seed maturation mutants are desiccation intolerant, making them suitable for investigating the underlying mechanisms of desiccation tolerance. The hypothesis that certain carbohydrates, e.g. oligosaccharides are crucial for the establishment of desiccation tolerance and therefore absent in desiccation intolerant

Table 7.2. Characteristics of wild-type (WT) *Arabidopsis* and seed maturation mutants. +, normal; -, lacking or altered; sens., sensitive; insens., insensitive; def., deficient; nd, not determined

Characteristic	WT	*aba1*[1,2] *aba2*[2] *aba3*[2]	*abi1*[3,4,5] *abi2*[3,4,5]	*abi3*[3,6,7]	*abi4*[8] *abi5*[8]	*rdo1*[9]	*rdo2*[9]	*fus3*[10,11,12]	*lec1*[12,13,14,15,16]	*lec2*[12]
Dormancy	+	-	-	-	+	-	-	-	-	+
Desiccation tolerance	+	+	+	+/-	+	+	+	-	-	+
Lipids	+	+	+	-	nd	nd	nd	-	-	-
Proteins	+	+	+	-	nd	nd	nd	-	-	-
ABA	sens.	def., sens.	insens.	insens.	insens.	sens.	sens.	sens.	sens.	nd
GA-inhibitor	sens.	insens.	insens. (*abi2* sens.)	insens.	nd	sens.	insens.	sens.	insens.?	nd
Seed colour	+(brown)	+	+	+/-(green)	+	+	+	-(purple)	-(purple)	-(purple)
Plant phenotype	+	wilty	wilty	+	+	short	+	+	+	+

[1] Koornneef et al., 1982
[2] Léon-Kloosterziel et al., 1996b
[3] Koornneef et al., 1984
[4] Finkelstein and Somerville, 1990
[5] Nambara et al., 1991
[6] Nambara et al., 1992
[7] Ooms et al., 1993
[8] Finkelstein, 1994
[9] Léon-Kloosterziel et al., 1996a
[10] Bäumlein et al., 1994
[11] Keith et al., 1994
[12] Meinke et al., 1994
[13] Meinke, 1992
[14] West et al., 1994
[15] Chapter 4
[16] Parcy et al., 1997

mutants could not be confirmed (Ooms et al., 1993; Chapter 4). Different desiccation tolerance-inducing treatments showed contradictory effects on seed carbohydrate composition and accumulation, indicating that carbohydrates might be involved, but are not the determinants of desiccation tolerance (Ooms et al., 1994). A possibility is to extend this kind of correlative analyses to other substances that might be important in desiccation tolerance. A drawback is that a causal correlation is sometimes difficult to prove. Moreover, mutants that are affected only in desiccation tolerance would also be preferable. The *emb270* (*embryo-defective*) mutant seems to be such a mutant (Vernon and Meinke, 1995). Further analysis of the many genes that have been isolated on the basis of their specific expression during seed development will most likely reveal that some of these are involved in the establishment of seed desiccation tolerance.

Seed dormancy

Seed dormancy is determined by characteristics of the two components composing the seed, the embryo and the seed coat. The *Arabidopsis* ABA-deficient mutants are good examples of mutants with an embryo-determined reduced dormancy (Karssen et al., 1983; Léon-Kloosterziel et al., 1996b). However, it is possible that also the testa plays a role in the reduced dormancy of the *aba1* mutant because it was found to have a reduced thickness of the mucilage layer (Karssen et al., 1983). This effect on the mucilage layer might depend on the genetic background because the *aba* mutants in Col background had a normal mucilage layer (unpublished results). In tomato, the ability of the ABA-deficient *sit* mutant to germinate on lower osmotic potential was attributed to a structural alteration in the mutant testa (Hilhorst and Downie, 1995).

Numerous studies using mutants or ABA biosynthesis inhibitors are known in which the role of ABA in the induction and maintenance of dormancy is investigated. In many species, ABA-deficiency leads to non-dormant seeds (Koornneef, 1986). The physiological analysis of the *Arabidopsis aba1* mutant showed that the induction of dormancy during seed development correlated with a peak in embryonic ABA and that maternal or applied ABA could not induce dormancy. The level of ABA in mature wild-type seeds is very low, and does not seem likely to be inhibitory for germination (Karssen et al., 1983). This may mean that after the induction of dormancy, ABA levels do not need to stay high in order to maintain dormancy. However, the presence of ABA can be responsible for the germination inhibiting effect in seeds, because in some species there is *de novo* ABA biosynthesis upon germination of dormant seeds. The ability to synthesize

ABA upon imbibition was suppressed after a dormancy-breaking period in sunflower seeds and in barley embryos that were non-dormant by a manipulation of the environment during maturation (Bianco et al., 1994; Wang et al., 1995). This indicates that *in situ* ABA biosynthesis may be necessary not only to induce, but also to maintain embryo dormancy. A role of ABA during imbibition is certainly in agreement with the non-dormant phenotype of the *Arabidopsis aba* and *abi* mutants, but also other possibilities have to be taken into account to explain the phenotypes of non-dormant mutants that are not ABA-deficient or -insensitive. For barley, a model is hypothesized that also includes the breakdown of extracellular ABA as a factor determining germination, because a non-dormant mutant showed an increased ability to degrade extracellular ABA (Visser et al., 1996). Most likely, mutants affecting ABA degradation are not present among the available *Arabidopsis* dormancy mutants because pleiotropic effects at other phases of either seed development (as in e.g. *abi3*) or at vegetative development (as in the case of *abi1* and *abi2*) may be expected. Furthermore, such mutants should also differ in their sensitivity to exogenously applied ABA, whereas the *rdo* mutants are only very slightly ABA-insensitive (Fig. 6.4).

All non-dormant ABA-related mutants have in common that they do not require GA for germination as was concluded from their resistance to GA biosynthesis inhibitors (Nambara et al., 1991; Léon-Kloosterziel et al., 1996b) and the presence of germination when such mutants are combined with non-germinating GA-deficient mutants (Koornneef et al., 1982; Nambara et al., 1992). The dormancy mechanism affected in these mutants therefore seems to be based on the induction of a GA requirement by ABA. The observation that other non-dormant mutants (*fus3*, *rdo1*, and possibly a number of dormancy genes identified in ecotypes) still retain a GA requirement for germination suggests that these genes control another dormancy or seed germination pathway that operates in parallel with the ABA/GA pathway.

The occurrence of coat imposed dormancy in *Arabidopsis* can be inferred from mutants affecting the testa. The *ats* (*aberrant testa shape*) mutant, in which the aberrant shape originates from altered integuments, is an example of a mutant with reduced dormancy due to an altered testa (Léon-Kloosterziel et al., 1994). The involvement of the testa in dormancy is also indicated by the *ap2* (*apetala*) mutant and the class of *tt* (*transparent testa*) mutants. The *AP2* gene is involved in the specification of floral organ identity and ovule development, leading to missing petals and an altered morphology of the outer layer of the testa in the mutant (Jofuku et al., 1994). Apparently, this leads to a reduced dormancy, because an *ap2*

mutant was found in a reduced dormancy screen applied on T-DNA mutagenized material (unpublished results). The *tt* mutants represent a rather extensive class of mutants of which several lack certain flavonoids in the testa, giving the seeds a yellow to light brown appearance. They have a normal seed shape but sometimes have a slightly altered testa structure. To date, mutants at at least 17 different loci have been identified, most of them showing a reduced dormancy (I.J. Debeaujon, personal communication). The biochemical characteristics of a number of *tt* mutants have been determined and reviewed by Shirley et al. (1995). Part of the mutant loci are structural genes that encode enzymes of the flavonoid biosynthetic pathway. Others may be regulators of flavonoid gene expression. Recently, additional *tt* mutants have been isolated and are analyzed in our laboratory. Some of these mutants have been isolated from T-DNA populations, enabling the cloning of the corresponding gene (I.J. Debeaujon, personal communication).

ABA biosynthesis

The ABA-deficient mutants at the new loci *aba2* and *aba3* have been included in the investigation of the ABA biosynthetic route. These studies have indicated that the *aba2* mutant is blocked in the conversion of xanthoxin into ABA-aldehyde and that the *aba3* mutant is unable to convert ABA-aldehyde into ABA (Figure 7.2). Lesions at this step may result from a defect in the aldehyde oxidase apoprotein or in a molybdenum cofactor (Moco), which the aldehyde oxidase requires. The *aba3* mutant lacked several additional enzyme activities, which require a Moco. The mutant was found to be affected in the insertion of sulfur into the Moco (Schwartz et al., 1997). Also in other plant species many ABA-deficient mutants are known, representing different steps in the biosynthesis route. The tomato *not* and the pea *wil* may be blocked in the cleavage step prior to xanthoxin but their characterization has not been sufficient to prove this (Taylor, 1991). In maize, molecular analysis of the *vp14* mutant indicated that this is a putative cleavage step mutant, because the gene shows sequence homology to bacterial dioxygenase enzymes. The *vp14* phenotype is not wilty, but is restricted to precocious germination of the seeds (McCarty, 1995; Tan et al., 1995). To date, no cleavage step mutants have been found in *Arabidopsis*. If, like in maize, these mutants show a seed-specific expression, screening for wilty phenotypes is not useful, but the emphasis should be on reduced dormancy mutants. This process is very sensitive to a reduction in ABA content as shown by the clear effects on dormancy in the leaky *aba1-3* allele (Karssen et al., 1983). Additional non-dormant mutants are presently analyzed and require the determination of ABA levels both in vegetative tissues and in seeds.

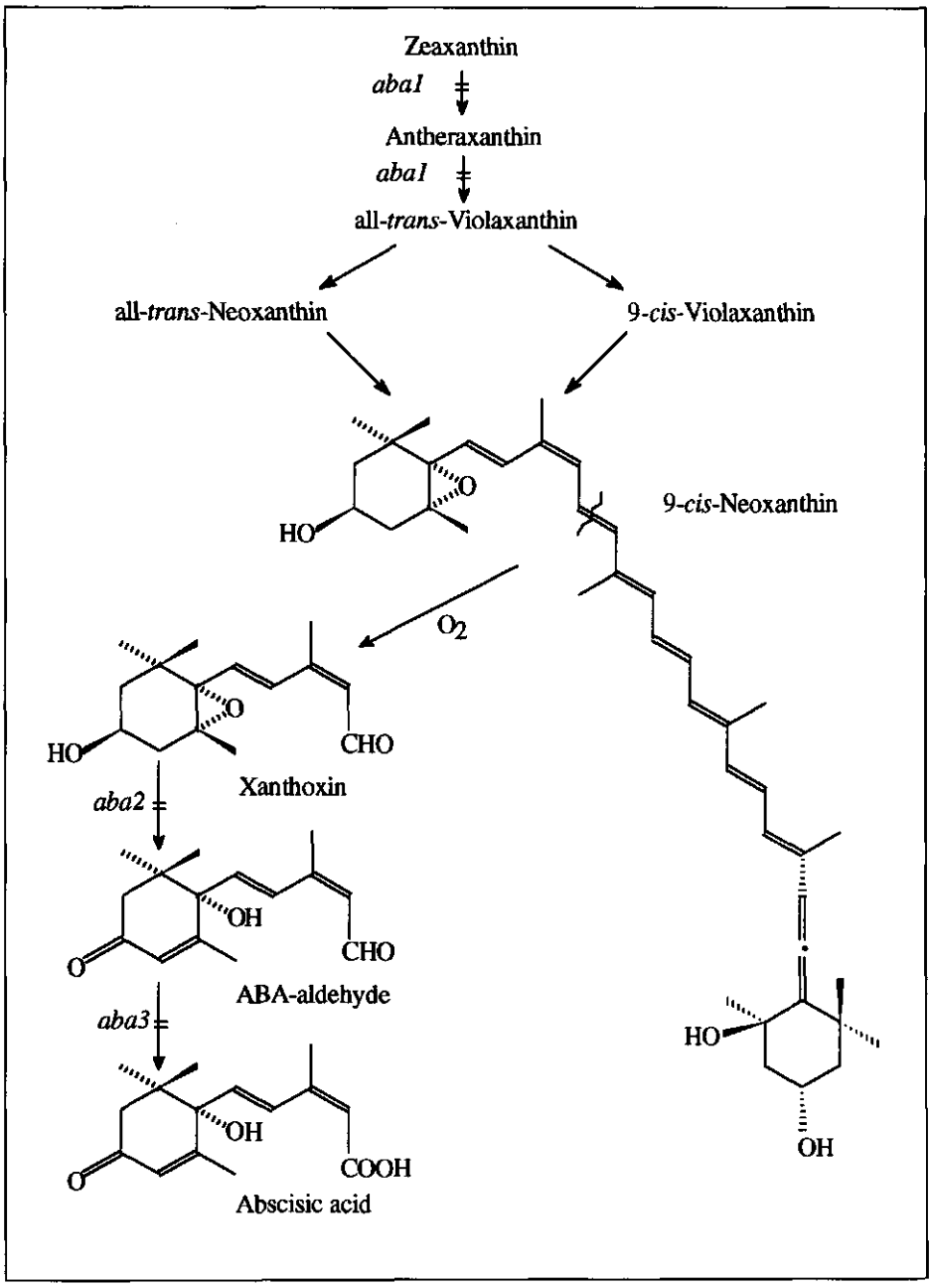

Figure 7.2. Biosynthesis pathway of ABA. The biochemical lesions in the *aba1*, *aba2*, and *aba3*, mutants are indicated.

So far, only one gene encoding an enzyme in the ABA biosynthetic pathway has been cloned. The *aba2* mutant of *Nicotiana plumbaginifolia* was isolated by transposon tagging. This led to the cloning of zeaxanthin epoxydase, which controls the first step of ABA biosynthesis. The complementation by the *N. plumbaginifolia ABA2* cDNA of both species' mutants confirmed that this mutation is homologous to the *Arabidopsis aba1* mutant (Marin et al., 1996). Cloned genes of the ABA biosynthetic pathway are of use for the study of the regulation of ABA biosynthesis and its cellular location and may give possibilities to manipulate dormancy or stress tolerance in crops.

Concluding remarks

Mutants are versatile tools in plant science. For example, hormone mutants have clarified much about the role of specific hormones in physiological processes. In addition, mutants enable the cloning of genes, which can lead to insight in the mode of action and the regulation of these processes. Genes can be cloned departing from mutants by positional cloning or by T-DNA or transposon tagging. Besides the use of mutants, other possible ways are open to isolate seed development genes. In the case of dormancy, molecular approaches have been described. For example, by screening of mRNAs that are differentially expressed in dormant and non-dormant *Avena fatua* embryos, several dormancy-associated cDNAs have been isolated. Some of these may encode LEA proteins; for many of the clones no functional relation to known genes has been found so far (Johnson et al., 1995; Li and Foley, 1995).

In addition to mutants, also genetic variation that is found in nature is amenable for genetic and molecular approaches. Complex and variable characteristics such as dormancy can be considered genetically as quantitative traits (determined by quantitative trait loci, QTL) and are difficult to analyse. Recently developed procedures using molecular markers and sophisticated mathematical procedures have improved the mapping of QTL tremendously (Jansen, 1996). For this, homozygous mapping populations such as recombinant inbred lines (RILs) are very informative because environmental effects can be reduced by means of replications. QTL analysis of the set of RILs derived from a cross between L*er* and Col identified 14 loci involved in seed germination, part of them independent from environmental factors and part of them only acting in very specific germination conditions (Van der Schaar et al., 1997). This analysis is being extended to a set of RILs between L*er* and Cvi (Cape Verde Islands). The latter ecotype shows a more profound dormancy than other ecotypes, leading to the identification of large-effect

dormancy loci by means of QTL analysis (C. Alonso-Blanco, personal communication). These loci can be introgressed into other ecotypes for genetic and physiological characterization. An accurate mapping of both induced mutants and quantitative trait loci will provide an important basis for the cloning of these genes, because positional cloning will be favoured by the international efforts that are being put into mapping and sequencing of the *Arabidopsis* genome. It might be worthwhile to start mutagenesis experiments in these very dormant genotypes, because it cannot be excluded that in these lines dormancy mechanisms are operating that are absent in the much less dormant ecotypes L*er* and Col. Furthermore, it will be important to continue the search for additional mutants especially those affecting specific components of the seed maturation process and those affecting the control of particular sets of seed maturation genes. For the latter the approach to select mutants with an altered expression of a reporter gene controlled by maturation-specific promoters looks very promising.

The study of the various mutants at the level of physiology and gene expression, their interaction and the molecular cloning of these genes, will increase our understanding of the processes that take place during the later phases of seed development.

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Samenvatting

Voor planten zijn zaden belangrijk voor de verspreiding en voor de overbrugging van perioden met ongunstige omstandigheden. De zaadontwikkeling bij de zandraket, *Arabidopsis thaliana* is het onderwerp van dit proefschrift. *Arabidopsis* wordt veel gebruikt in het botanisch genetisch onderzoek omdat het een zelfbevruchtende soort is met een korte levenscyclus. Bovendien nemen de planten weinig ruimte in en zijn gemakkelijk te kweken en te kruisen. Het heeft een overzichtelijke DNA-structuur in slechts 5 chromosoomparen, dat voor een belangrijk deel in kaart gebracht is.

De zaadontwikkeling bij *Arabidopsis* duurt ongeveer 3 weken. In de eerste anderhalve week ontwikkelt het zaad zich totdat het is volgroeid en bestaat uit een embryo en een zaadhuid. Hierna begint het stadium van de zaadrijping, hierin vinden er belangrijke fysiologische veranderingen plaats. Dit zijn ten eerste het tot stand komen van kiemrust en uitdrogingstolerantie. Kiemrust is de toestand van het zaad waarin het wel levensvatbaar is, maar toch niet kiemt na het zaaien ook al zijn de omstandigheden gunstig (voldoende lucht, licht en water). De kiemrust is opgeheven na een aantal weken droge bewaring van de zaden. Ook een koubehandeling na het zaaien kan de kiemrust opheffen. Een zaad moet uitdrogingstolerant zijn omdat het slechts weinig water bevat, terwijl het embryo wel levensvatbaar moet blijven. Tijdens de zaadrijping worden ook eiwitten en vetten, die dienen als reservevoedsel na de kieming, aangemaakt.

Het doel van dit onderzoek was om na te gaan welke genen een rol spelen bij de zaadontwikkeling en in het bijzonder bij de zaadrijping. Een gen is een functionele eenheid van DNA. Als er een mutatie in het gen zit, is het gen niet meer of slecht gedeeltelijk functioneel en kan een afwijking geven die zichtbaar en/of meetbaar is aan de plant. Een plant met één of meer "kapotte" genen noemen we een mutant en het afwijkende uiterlijk het mutante fenotype. Het mutante fenotype zegt iets over de functie van het desbetreffende gen. Om de genen van de zaadontwikkeling te bestuderen werden eerst de mutanten geselecteerd na een behandeling met een mutagene stof of straling. De zaadontwikkeling omvat vele aspecten waaronder bijvoorbeeld kiemrust. Eén van de mutantselecties betrof de selectie op verminderde kiemrust waarvoor de zaden van de behandelde planten direct na het oogsten werden gezaaid. Normale ("wildtype") zaden zijn dan in kiemrust en zullen niet kiemen. Zaden die dan toch kiemen hebben dus een verminderde kiemrust en hebben waarschijnlijk een mutatie in een gen dat (mede)verantwoordelijk is voor het tot stand komen van kiemrust.

De geselecteerde mutanten werden genetisch gekarakteriseerd. Dit betekent dat de overerving van het mutante gen en de plaats op één van de chromosomen werd bepaald. Ook kan de interactie tussen twee genen bestudeerd worden door te kijken wat er gebeurt met een plant die twee mutante genen heeft (dubbelmutant), die er door middel van kruising ingebracht zijn.

In een selectie op verminderde kiemrust werd een mutant (*ats*-mutant) gevonden met een afwijkende zaadvorm, hartvormig in plaats van langwerpig (hoofdstuk 2). Deze mutatie bevond zich op chromosoom 5. Genetische en electronen-microscopische analyse van deze mutant liet zien dat de zaadhuid een afwijkende vorm had en daardoor ook het embryo in een andere vorm dwingt. Ook de verminderde kiemrust werd veroorzaakt door de afwijking aan de zaadhuid. Deze afwijking ontstond al voor de bevruchting, tijdens de ontwikkeling van de zaadknop. Het mutante gen speelt dus een rol in de ontwikkeling van de integumenten, de buitenste cellagen van de zaadknop.

Het was al bekend dat het plantenhormoon abscisinezuur (ABA) een belangrijke rol speelt tijdens de zaadrijping. Behalve op de zaadrijping heeft ABA ook effect op de waterhuishouding van de plant en reacties van de plant op stress, zoals droogte en kou. Er zijn twee soorten ABA-mutanten, ten eerste ABA-deficiënte mutanten (*aba*-mutanten) die een mutatie hebben in een gen dat betrokken is bij de aanmaak van ABA, waardoor het ABA-gehalte sterk verlaagd is. Ten tweede zijn er de ABA-ongevoelige (o.a. *abi3*) mutanten, die een normaal ABA-gehalte hebben, maar waarvan de zaden niet op ABA reageren. Het plantenhormoon gibberelline (GA) heeft een werking die tegengesteld is aan die van ABA. Zaden hebben GA nodig voor de kieming en mogelijk is de gevoeligheid voor GA betrokken bij het doorbreken van kiemrust. Behalve de genoemde selecties op verminderde kiemrust zijn er in dit onderzoek ook andere selecties toegepast. Zo is er gezocht naar groene zaden. Ook is er geselecteerd op zaden die in staat waren te kiemen op GA-remmers en een hoge concentratie ABA, waarop wildtype zaden niet kiemen. Verschillende selecties hebben zowel nieuwe ABA-deficiënte als nieuwe ABA-ongevoelige mutanten opgeleverd. ABA-deficiënte mutanten (hoofdstuk 5) vertonen een verminderde kiemrust en hebben hierdoor minder GA nodig voor de kieming. De kieming van deze zaden blijkt namelijk relatief ongevoelig te zijn voor een stof die de aanmaak van GA remt.

In mutanten die extreem ongevoelig zijn voor ABA (*abi3*-mutanten) is de zaadrijping ernstig verstoord. De zaden zijn groen i.p.v. bruin, hebben geen kiemrust en uitdrogingstolerantie en missen veel van de reservevoedsel-eiwitten. Ook de zaden van de *fus3*- en *lec1*-mutanten hebben deze afwijkingen, maar deze

zijn donkerrood en die van de *fus3*-mutant zijn niet ABA-ongevoelig. Omdat bepaalde koolhydraten een rol zouden kunnen spelen bij uitdrogingstolerantie is nagegaan of bovengenoemde mutanten een andere koolhydraat-samenstelling hebben (hoofdstuk 3 en 4). Bij vergelijking van de koolhydraat-samenstelling van de mutanten met het wildtype bleken er geen specifieke koolhydraten te zijn die een rol spelen bij het bewerkstelligen van uitdrogingstolerantie. Wel was het totaal koolhydraat-gehalte hoger in de mutante zaden, waarschijnlijk ter compensatie van het gebrek aan reservevoedsel-eiwitten en -vetten.

Er is nagegaan in hoeverre kiemrust en kieming in de *abi3*, *lec1* en *fus3* mutanten afhankelijk is van GA. Hiervoor zijn dubbelmutanten gemaakt die zowel één van de zaadrijpingsmutaties als een GA-deficiënte mutatie hadden. Een GA-deficiënte mutant kan niet kiemen omdat deze geen GA aanmaakt wat nodig is voor de kieming. De *abi3*- en *lec1*-mutanten die ook een GA-deficiënte mutatie hadden, kiemden wel, wat betekent dat deze minder GA nodig hebben om te kiemen. Dit in tegenstelling tot de *fus3*-mutant die wel GA nodig had voor de kieming. In deze mutanten hangt deze GA-behoefte dus samen met de ongevoeligheid voor ABA.

Behalve bovengenoemde mutanten zijn er ook twee mutanten geïsoleerd (*rdo*-mutanten) die wel een verminderde kiemrust hadden maar niet ABA-deficiënt of ABA-ongevoelig waren (hoofdstuk 6). Evenmin leken deze mutanten een veranderde gevoeligheid te hebben voor andere hormonen, die soms bij de kieming ook een rol spelen zoals ethyleen, auxine en cytokinine. Een zaadeigenschap zoals kiemrust komt tot stand in een groot aantal stapjes en bij elk stapje is weer een ander gen betrokken (regulatieketen). Ook kunnen delen van de regulatieketen parallel lopen. Twee genen met elk een klein effect op de kiemrust, zoals die in de beide *rdo*-mutanten, geven samen een groter effect, wat te zien is aan een sterker verminderde kiemrust in de dubbelmutant van deze twee. Dit betekent dat deze genen zich waarschijnlijk elk in een parallel gedeelte van de regulatieketen bevinden. De genen die betrokken zijn bij de aanmaak en gevoeligheid voor ABA bevinden zich waarschijnlijk ergens in het begin waarna de regulatieketen zich vertakt. De *RDO* genen bevinden zich dan waarschijnlijk ergens aan het einde, omdat deze alleen een effect op de kiemrust en niet op andere aspecten van de zaadrijping hebben.

In vervolgonderzoek kunnen mutanten op verschillende manieren nuttig zijn. Vaak kan uitgaande van een mutant het bijbehorende gen geïsoleerd worden met behulp van verschillende moleculair biologische technieken. Soms kan dat leiden tot praktische toepassingen. Een gen dat bijvoorbeeld betrokken is bij de aanmaak van ABA kan mogelijk gebruikt worden om kiemrust en stresstolerantie te

manipuleren in gewassen. Maar vooral zijn deze genen van belang om antwoord te krijgen op vragen van fundamentele aard. Door de interactie van genen te analyseren in dubbelmutanten of door te kijken waar en onder welke voorwaarden (bijvoorbeeld in combinatie met een andere mutant) een geïsoleerd gen tot expressie komt, kan antwoord gekregen worden op de vraag op wat voor manier al deze genen samenwerken om uiteindelijk tot een rijp zaad te komen.

Publications

Part of this thesis has been published in journals:

Ooms, J.J.J., **Léon-Kloosterziel, K.M.**, Bartels, D., Koornneef, M., Karssen, C.M. (1993) Acquisition of desiccation tolerance and longevity in seeds of *Arabidopsis thaliana*. *Plant Physiol.* 102:1185-1191 (Chapter 3)

Léon-Kloosterziel, K.M., Keijzer, C.J., Koornneef, M. (1994) A seed shape mutant of *Arabidopsis* that is affected in integument development. *Plant Cell* 6:385-392 (Chapter 2)

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Léon-Kloosterziel, K.M., Alvarez Gil, M., Ruijs, G.J., Jacobsen, S.E., Olszewski, N.E., Schwartz, S.H., Zeevaart, J.A.D., Koornneef, M. (1996) Isolation and characterization of abscisic acid-deficient *Arabidopsis* mutants at two new loci. *Plant J.* 10:655-661 (Chapter 5)

Related papers not presented in this thesis:

Schwartz, S.H., **Léon-Kloosterziel, K.M.**, Koornneef, M., Zeevaart, J.A.D. (1997) Biochemical characterization of the *aba2* and *aba3* mutants in *Arabidopsis thaliana*. *Plant Physiol.* 114:161-166

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Nawoord

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Curriculum vitae

Karen Marit Kloosterziel werd geboren te Hengelo (Ov.) op 10 mei 1967. In augustus 1979 ging zij naar het dr. F.H. de Bruijne Lyceum in Utrecht, alwaar in juni 1985 het VWO-diploma behaald werd. Zij studeerde biologie aan de Vrije Universiteit (VU) te Amsterdam van september 1985 tot maart 1991. De doctoraalfase van de studie bestond uit onderzoeksstages plantengenetica en fytopathologie bij de werkgroep Somatische Celgenetica aan de VU, het Fytopathologisch Laboratorium "Willie Commelin Scholten" te Baarn en het Centrum voor Plantenveredeling (tegenwoordig CPRO) te Wageningen. Van maart 1991 tot maart 1996 was ze als AIO in dienst van de Landbouwuniversiteit Wageningen, waar ze onder begeleiding van prof. dr. ir. M. Koornneef werkte aan zaadontwikkelingsmutanten van *Arabidopsis*. De resultaten van dit werk zijn beschreven in dit proefschrift.