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Sequential steps for developmental arrest in *Arabidopsis* seeds

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SUMMARY

The continuous growth of the plant embryo is interrupted during the seed maturation processes which results in a dormant seed. The embryo continues development after germination when it grows into a seedling. The embryo growth phase starts after morphogenesis and ends when the embryo fills the seed sac. Very little is known about the processes regulating this phase. We describe mutants that affect embryo growth in two sequential developmental stages. Firstly, embryo growth arrest is regulated by the FUS3/LEC type genes, as mutations in these genes cause a continuation of growth in immature embryos. Secondly, a later stage of embryo dormancy is regulated by ABI3 and abscisic acid; *abi3* and *aba1* mutants exhibit premature germination only after embryos mature. Mutations affecting both developmental stages result in an additive phenotype and double mutants are highly viviparous. Embryo growth arrest is regulated by cell division activities in both the embryo and the endosperm, which are gradually switched off at the mature embryo stage. In the *fus3/lec* mutants, however, cell division in both the embryo and endosperm is not arrested, but rather is prolonged throughout seed maturation. Furthermore ectopic cell division occurs in seedlings. Our results indicate that seed dormancy is secured via at least two sequential developmental processes: embryo growth arrest, which is regulated by cell division and embryo dormancy.

Key words: Embryo growth, Growth arrest, Embryo dormancy, *fus3*, *lec*, *Arabidopsis thaliana*

INTRODUCTION

Multicellular organisms require proper timing for control of their development. This requires the correct timing of cell division, cell-fate commitment and differentiation, which in plants determines the phase transition between the embryo, the seedling and the adult organism. Phase transitions imply changes of cell division rates and patterns, leading to changes in plant growth rate and the formations of new structures. Entering a new phase requires a change in the balance of expression of numerous genes (Kerstetter and Poethig, 1998). How developmental timing is controlled is still not well understood.

Seed development comprises two major phases: embryo development and seed maturation. Embryogenesis starts with a morphogenesis phase and ends at the heart stage when all embryo structures have been formed (Mayer et al., 1991). It is followed by a growth phase during which the embryo fills the seed sac (Goldberg et al., 1994). At the end of embryo growth phase, cell division in the embryo arrests. The signals that induce cell division arrest and in what way arrest triggers the initiation of the following maturation phase is not known. Later during development the seed, containing a full size embryo, undergoes maturation during which food reserves accumulate and dormancy and desiccation tolerance develop (Goldberg et al., 1994). The change from embryogenesis to maturation is characterized by an interruption of growth and is associated with changes of cell growth activity and gene expression.

During normal seed development embryo arrest is reversed upon germination, when proper environmental conditions are provided and the dry seeds imbibe water. Germination, or breaking of dormancy, is initially characterised by cell elongation, which allows the embryo to break through the surrounding envelopes (Mansfield and Briarty, 1996). After germination, during seedling growth, cell division resumes.

Seed development has been extensively studied in *Arabidopsis* using mutants defective in various aspects of the process. Mutants affecting the morphogenesis phase result in lethality or seedlings exhibiting abnormal phenotypes (Mayer et al., 1991). Seed maturation mutants, however, exhibit a normal seedling phenotype, but properties of germination and dormancy, as well as desiccation tolerance are defective (Koornneef and Karssen, 1994; Goldberg et al., 1994).

Two major groups of seed maturation genes, differing in the number of pleiotropic effects they have, can be recognised (Holdsworth et al., 1999). The first group includes loci that have a broad effect during seed maturation such as the *ABI3*, *FUS3* and *LEC* loci. In *abi3*, *lec1* and *fus3* mutants the accumulation of storage compounds, various proteins, chlorophyll and anthocyanins, dormancy and desiccation tolerance are defective (Holdsworth et al., 1999; Wobus and Weber, 1999). The *lec1*, *lec2* and *fus3* mutants differ

morphologically from the *abi3* mutants as they form trichomes on their cotyledons, which are also absent in wild type. Although the master regulators *FUS3*, *LEC1* and *ABI3* have been cloned is it not clear how they function during seed development. One possibility is that they function together as transcription factors and can form hetero-oligomeric combinations that regulate various maturation-associated genes (Parcy et al., 1997; Wobus and Weber, 1999). The second group contains mutants with diverse defects in restricted aspects of seed maturation, an example of which is the *wrinkled* seed mutant (Focks and Benning, 1998) defective in seed storage accumulation. In addition, mutants that do not germinate such as *comatose* (Russell et al., 2000) and *sly* (Steber et al., 1998) or mutants with reduced seed dormancy (Léon-Kloosterziel et al., 1996) can also be classified in this second group. Among the seed germination and dormancy mutants are those that also affect additional stages of plant development. These include abscisic acid (ABA; Koornneef et al., 1998) and gibberellin (GA) biosynthesis mutants (Koornneef and van der Veen, 1980), and those with an altered testa pigmentation or structure (Debeaujon et al., 2000). The study of ABA and GA mutants in *Arabidopsis* and maize has shown that, during germination, GA antagonises ABA signalling (Koornneef et al., 1998; White and Rivin, 2000). This dormancy has both embryo and testa components. Without ABA and also when the seed coat is mechanically removed or genetically weakened, seeds do not require GA for germination (Debeaujon and Koornneef, 2000).

While the processes and genes regulating morphogenesis and maturation are being identified, little is known about the embryo growth phase. We have used previously described mutants to study the embryo growth phase. Detailed analyses of seed maturation mutants in relation to developmental timing during the embryo growth phase, together with assessment of cell cycle activity allows us to suggest two distinct developmental processes during seed development. We show that the *fus/lec* mutants affect embryo growth as early as the walking-stick stage, and the cell cycle during later stages. The *abi3* and *aba1* mutants, however, do not affect cell division but result in premature germination during early maturation phase, in addition to their effect on dormancy observed during germination of mature seeds.

MATERIALS AND METHODS

Plant material and growth conditions

The mutants *fus3-8, abi3-6* (Nambara et al., 1995, provided by P. McCourt), *aba1-5* and *tt3-3* (provided by I. Debeaujon) are in the Colombia (Col) background. The irradiation-induced *fus3-8* mutant was isolated in a screen for premature germination, and subsequently backcrossed three times to Landsberg *erecta* (L*er*) to obtain the *fus3-8/Ler* line. The *lec2-1* (Meinke et al., 1994), originally in the WS1 background, provided by David Meinke, and the *fus3-2* mutant, a gift from Simon Misera (Baumlein et al., 1994) in the Dijon background were both backcrossed five times with L*er* so that they were considered near-isogenic lines. The mutants *ga1-3, lec1-3, abi3-5* and *aba1-1* are all in the L*er* background. The *lec1-3* mutant was selected by Peeters and Koornneef (unpublished) from an En/I transposon-containing L*er* population (Aarts et al., 1995). The Cyc1B-GUS transgenic line FA4C in the Col background (Colon-Carmona et al., 1999) was provided by Peter Doerner. For double mutant analyses mutants from the same background were crossed. Plants were grown in an air-conditioned greenhouse at 18-25°C with additional light provided in winter (at least 16 hours photoperiod from Philips HP1-T/400 W lamps). For premature germination studies immature siliques were harvested, cut open and plated on 0.4% water-agar plates. Germination and growth experiments were carried out under continuous light at 22-24°C.

GUS staining and imaging

GUS staining was done as described by Raz and Koornneef (Raz and Koornneef, 2001). Microscopic analyses were carried with Nomarski optics using a Nikon Optiphot microscope. For confocal microscope analyses immature seeds were stained using the Feulgen staining method (Siddiqi et al., 1999) and analysed with a laser confocal microscope (Bio-Rad laser, CoMOS 7 operating software).

Western blot analysis

Protein extractions were carried out in PBS+1% Tween. Protein sample buffer was added and proteins were boiled for 5 minutes. The extraction mix was centrifuged for 5 minutes and supernatant was transferred into a new tube. Aliquots of proteins were separated on 10% SDS-PAGE using the Bio-Rad mini system. Blotting and immunodetection with HRP reagents (ECL) was carried according to the Bio-Rad protocol. Membranes were stained with Ponceau-S (Sigma). KNOLLE protein was detected with anti-KNOLLE antibody (Lauber et al., 1997), β-tubulin was detected with monoclonal antibodies (Sigma).

Flow cytometry

For cell cycle analyses, suspensions of intact nuclei were prepared from fresh seeds (Arumuganthan and Earle, 1991). Samples of 50-30 seeds were chopped with a razor blade in ice-cold nucleus-isolation buffer (10 mM $MgSO_4.7H_2O$, 50 mM KCl, 5 mM Hepes, 1 mg/ml DTT and 2.5 mg/ml Triton X-100, 1% (w/v) PVP-40) on ice. Each seed batch was measured in duplicate. After chopping, the suspension was passed through an 88 μm nylon mesh and stained with propidium iodide (1 mg /ml) for 10 minutes. DNA analyses were performed with a Beckman-Coulter EPICS XL-MCL flow cytometer (Beckman-Coulter, Miami, FL, USA) equipped with an argon ion laser at 488 nm. The amount of DNA, proportional to the red fluorescent signal, is expressed as arbitrary C values in which 1C represents the amount of DNA of the unreplicated haploid chromosome complement. The number of nuclei present in each peak of the histogram, 2C, 3C, 4C, 6C and 8C, was analysed by measuring the peak area. Histograms were processed using ModFit LT (Verity, USA) for data analysis and correction of the background noise, and the volume of each histogram was calculated. The percentage of nuclei at S phase and G_2 in the embryo was calculated as 4C+8C/(2C+4C+8C). The percentage of nuclei in the endosperm was calculated as 3C+6C/(2C+3C+4C+6C+8C).

RESULTS

DNA replication and mitotic activity during embryo growth phase

Embryo pattern formation ends at the heart stage, after which all embryonic tissues have been differentiated. Subsequently, the embryo growth phase starts and takes about 4-5 days. The morphologically recognisable steps are: torpedo, walking-stick, bent cotyledons, mature and full size, during which the embryo enlarges until it fills the seed (Fig. 1A). To characterise growth during the growth phase we analysed the profile of cell divisions in developing wild-type seeds (Fig. 1). The expression of the mitotic cyclin, Cyc1B is restricted to cells

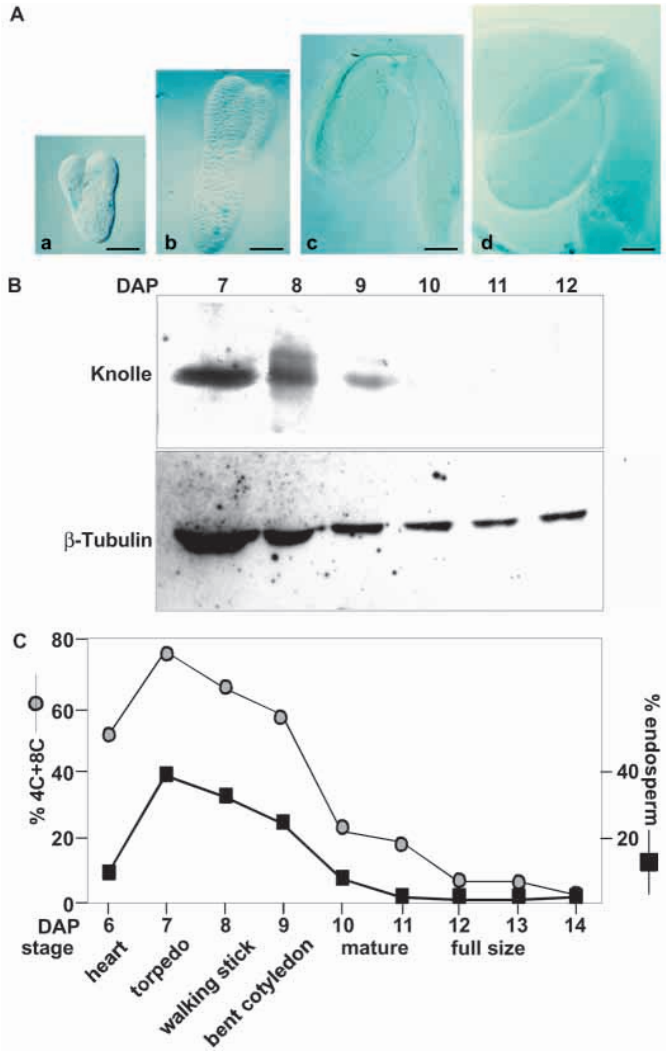


Fig. 1. Profile of cell division during embryo growth phase. (A) Cyc1B-GUS expression during embryo growth phase in immature seeds from Cyc1B-GUS transgenic plants. a, early torpedo; b, early walking-stick; c, bent-cotyledon. d, full size. (B) Expression of KNOLLE and β-tubulin proteins during embryo growth phase. Western blot analyses of KNOLLE and β-tubulin proteins in immature seeds 7-12 DAP. (C) DNA content in seeds during embryo growth phase. Seeds were analysed for their DNA content, and the percentage of embryo nuclei with replicated DNA (4C+8C in the embryo and in the endosperm, 3C+6C) was calculated as described in Methods. Scale bars, 15 μm.

undergoing mitosis, and Cyc1B-GUS reporter gene activity was used as a molecular marker for mitotic cells (Colon-Carmona et al., 1999; Raz and Koornneef, 2001). Throughout the torpedo and walking-stick stages GUS expression was detectable in all parts of the embryo. During the bent-cotyledon stage the number of GUS-expressing cells declined and at the mature stage GUS expression was not detectable (Fig. 1A).

Expression of the *KNOLLE* gene is restricted to mitotic cells and the protein is localized to the mitotic cell plate (Lauber et al., 1997). During the embryo growth phase KNOLLE protein was detected 7-9 DAP (days after pollination) but was no longer detectable by 10 DAP, when the embryo was close to the seed maturation phase (Fig. 1B). In tomato seeds, β-tubulin expression correlated with the presence of mitotic cells (de Castro et al., 1995). In *Arabidopsis* embryos expression gradually decreased between the torpedo and mature stages, consistent with its expression in tomato embryos. In *Arabidopsis*, however, β-tubulin expression remained steady during early seed maturation (Fig. 1B).

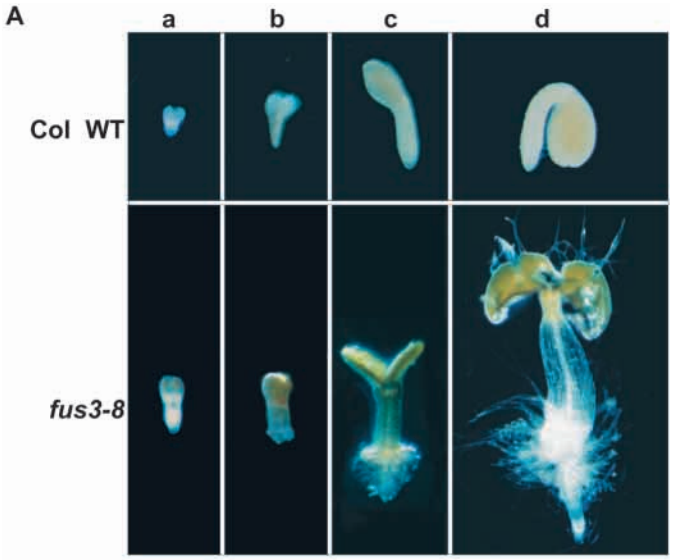
The profile of DNA content in the developing seed was determined using FACS analyses. Histograms of seed nuclei contained 2C, 4C and 8C, which are derived from embryo cells, and 3C and 6C, which are derived from the endosperm. DNA content in the embryo can indicate mitotic activity as the 2C value can be found in nuclei after mitotic division and in G_1 arrest while cells in which DNA has replicated have two or four times the amount of DNA. The relative content of 4C+8C to total embryo nuclei was found to increase between the heart and torpedo stages (Fig. 1C), during early growth phase. However, the percentage of nuclei with 4C+8C decreased as the growth phase proceeded and embryo cells were in mitotic arrest. This arrest continued throughout seed maturation, and mitotic activity resumed after germination (not shown). The relative amount of endosperm DNA was calculated relative to total amount of DNA in the seeds. The profile of the relative amount of endosperm DNA followed the profile of mitotic activity in the embryo (Fig. 1C); by the end of the growth phase most endosperm was degraded.

These results indicate that during the embryo growth phase mitotic activities in both the embryo and the endosperm increase up to the torpedo-walking stick stages and become arrested at the mature stage. This raises the question of whether the embryo is actively signalled to arrest growth. Mutants affected in this step are then expected to exhibit reduced embryo growth arrest. We screened for mutants that potentially exhibited a reduced embryo growth arrest and as a consequence directly developed into a seedling. For these experiments, described hereafter, we used novel alleles for *fus3-8* (both Col and L*er* genetic backgrounds) and *lec1-3* (L*er*) together with previously described alleles of *lec2-1* (L*er*), *abi3-5* (L*er*) and *abi3-6* (Col) and *aba1-1* (L*er*) and *aba1-5* (Col), all are strong alleles based on the mutant phenotypes.

Cell-division activity in mutants defective in embryo growth arrest

To study the growth capacity of the embryo, embryos from the heart stage, at 6 DAP, until the early seed maturation phase, 12 DAP, were excised from the seed and plated on water-agar plates. Col or L*er* wild-type embryos at these stages showed no growth capacity under these conditions, the embryos remained the same size and did not accumulate chlorophyll (Fig. 2A). After a week the embryo tissue disintegrated (not shown). In contrast, in *fus3-8* the growth capacity of embryos was not arrested (Fig. 2A). Four days after excision, embryos at the torpedo stage increased somewhat in size and chlorophyll accumulated. The growth capacity of the embryo increased and by the bent-cotyledon stage the embryo could grow into a healthy seedling that will complete its life cycle when planted in soil. When excised during the heart stage neither *fus3-8* embryos nor other mutants in both Col and L*er* genetic backgrounds exhibited subsequent growth (Fig. 2B). Both *lec1-3* and *lec2-1* mutants embryos had the ability to grow from, respectively, the walking-stick and bent-cotyledon stages onwards. The growth capacity of *abi3-5* and *aba1-1* embryos remained arrested during the growth phase. However full size

A



B

Genotype	DAP (stage) 6 (heart)	7 (torpedo)	8 (stick)	9 (bent)	10 (mature)	11 (full size)	12 (full size)
Ler WT	–	–	–	–	–	–	–
*fus3-8**	–	+	+	+	+	+	+
lec1-3	–	–	+	+	+	+	+
lec2-1	–	–	–	+	+	+	+
abi3-5	–	–	–	–	–	+	+
aba1-1	–	–	–	–	–	–	– (+14 DAP)

Fig. 2. Embryo growth arrest and embryo growth. (A) Embryos were excised from seeds and plated on water-agar. Photographs of Col wild type (upper panel) or *fus3-8* (lower panel) were taken 4 days after plating. Embryo stages: a, early torpedo; b, early walking-stick; c, early bent-cotyledon; d, mature, were determined at time of embryo excision. (B) Embryo growth in mutants. Seeds 6-12 DAP were harvested and embryos were excised from seeds. Embryo stage was determined and the embryos were plated on water-agar. Growth was determined after 4 days: –, indicates no growth, as observed in the wild type; +, indicates growth, as observed in *fus3-8*. Premature germination of *aba1-1* was observed in seeds 14 DAP (+14). 'Stick' and 'bent' in the table refer to the stages walking-stick and bent-cotyledons respectively. *fus3-8** is *fus3-8/Ler*.

embryos of these mutants excised during early seed maturation phase at 11 and 14 DAP respectively grew into seedlings (Fig. 2B). These experiments show that *fus3*, *lec1* and *lec2* can be classified as embryo growth arrest mutants. Although this phenotype can be detected as early as torpedo and walking-stick stages, a clearer phenotype was observed during the bent-cotyledon stage. Under the conditions used these mutant embryos can grow into seedlings with a normal axis, indicating that the morphogenesis phase is not defective.

Cell division activity in the embryo is prolonged in *embryo growth arrest* mutants

By the end of the normal embryo growth phase cell division, as well as embryo growth, is arrested. To understand further how cell division is involved in embryo growth arrest, cell division was studied in *embryo growth arrest* mutants. The DNA content of seeds was analysed 10 DAP. To ensure that the developmental stage was similar between wild type and mutants, seeds were isolated from heterozygous plants and sorted for the morphologically recognisable mutant seeds. In *fus3, lec1* and *lec2* mutants DNA replication continued in the embryo after the time that it was arrested in wild type (Fig. 3A). Compared with earlier phases the fraction of 4C + 8C nuclei in *fus*/*lec* mutants was reduced during the maturation phase but it remained higher than in wild type throughout seed maturation (Fig. 3B).

Nuclei with 16C and 32C content were detected in *fus3-8*, *lec1* and *lec2* mutants (not shown). These endoreplicated nuclei probably indicate the presence of trichomes on cotyledons in these mutants as trichomes contain endoreplicated nuclei (Walker et al., 2000). The DNA content in *abi3* and *aba1* showed no significant difference to that in wild type embryos (Fig. 3A,B). The expression of KNOLLE and β-tubulin proteins was also studied in these mutants. Both proteins accumulated normally during early growth phase and their expression stopped at maturity in wild-type and *abi3* embryos (Fig. 3C). In the *fus3* mutant the accumulation of these proteins was similar to that in wild type during early growth phase but continued during the mature stage, 10 DAP (Fig. 3C). During the growth phase the embryo remains green, thus we have used the RUBISCO protein as a loading control. Accumulation of these proteins in *lec1* and *lec2* showed the same pattern as in *fus3* (not shown). To study the expression of the mitotic Cyc1B-GUS in *fus3-8* and *abi3-6* mutants, in the Col background, these mutants were crossed with the transgenic line Cyc1B-GUS (Colon-Carmona et al., 1999). GUS staining was observed in mature *fus3* embryos, whereas in mature wild-type or *abi3* (not shown) embryos GUS staining could not be detected (Fig. 3D).

Histological analyses of *fus3-8* using confocal microscopy and Nomarski optics showed normal development from the heart to walking-stick stages onwards. Seeds from 7 DAP undergo oxidation and browning of the seed coat that interferes with histological analyses, so the *fus3-8* embryos were studied in the *transparent testa* (*tt3*) mutant background (Shirley et al., 1995). The *tt3* mutant has no effect on embryo development but the testa remains transparent during seed development allowing histological observations. During the bent cotyledon stage, ectopic cell division could be observed in the *fus3* embryo. These ectopic cell divisions occurred randomly, and as a result, the morphology of mature embryos was variable (Fig. 3Eb,d). In many embryos additional cell files could be observed in hypocotyls (Fig. 3Ed), in some embryos the shoot apical meristem was enlarged (Fig. 3Eb). Despite the enhanced cell division activity in *fus3*, the embryo was not larger than wild-type embryos (Fig. 3Eb and 3Ea, respectively). Moreover, sometimes *fus3* embryos exhibited reduced cotyledon size and mature embryos exhibited a walking-stick-like morphology (Fig. 3D). While in wild-type seeds, as well as in *abi3* and *aba1* seeds the endosperm is degraded at the end of the growth phase in *embryo growth arrest* mutants the endosperm degradation was reduced (Figs 4A, 3B). Using confocal sections, an endosperm with several cell layers was observed in mature *fus3* embryos, while in the wild-type embryo from the same silique the endosperm was restricted to one cell layer (Fig. 4B).

Thus in *embryo growth arrest* mutants cell division activities in both the embryo and the endosperm are not terminated by the end of the growth phase. In addition, during the growth phase, ectopic cell division in the *fus3* embryo resulted in a

slightly aberrant morphology, although the embryo and seedling axis remained intact.

Premature germination and the role of gibberellins

We tested our mutants exhibiting an embryo growth phenotype to determine whether they also germinated prematurely. When siliques were detached from the plant, cut open and placed on water agar, or MS medium, mutant embryos inside the seed coat germinated prematurely, while wild-type seeds did not germinate (Fig. 5A). *fus3-2*, *lec1-3* and *lec2-1*, however, exhibited premature germination in detached siliques from 8-9 DAP (Fig. 5B). *abi3-5* and *aba1-1* seeds exhibited premature germination only 10-11 and 14 DAP, respectively (Fig. 5B). Later during seed development the profile of premature germination in the single mutants remained similar (Fig. 6A). Gibberellins (GAs) are required for germination and release from dormancy of dormant seeds (Koornneef and van der Veen, 1980), thus *ga1-3* mature seeds show strongly reduced germination. Using a genetic approach we studied GA requirements during premature germination. Mutants exhibiting premature germination were crossed into *ga1-3*, and double mutants were selected based on the seed phenotype of the embryo arrest mutants and the dwarfness of the *ga1* mutant. The *ga1-3* mutant did not germinate prematurely (Fig. 6B). Premature germination of *fus3-2* and *lec2-1* was dependent on GA, as double mutants exhibited poor germination (Fig. 6B) compared with the parents (Fig. 6A). Premature germination of *abi3* and *aba1* also required GAs (Fig. 6B), however this requirement is not as strong as that of *fus3* and *lec2*. For *abi3-5* it was surmounted after a longer incubation period as *ga1-3 abi3-5* showed a higher germination percentage when scored after 7 days. Premature germination of *lec1-3*, however, is GA independent as shown by the germination of the *ga1-3 lec1-3* double mutant (Fig. 6B).

These results indicate that premature germination can be induced by at least two pathways: one, which is GA-dependent and involves *FUS3, LEC2, ABI3* and *ABA1*, and another GA-independent pathway involving *LEC1*. The ABA/GA ratio that initially inhibits premature germination may be changed during incubation to overcome the GA requirement for premature germination in *abi3*.

Double mutant analyses and *vivipary*

The *fus3, lec1* and *lec2* mutants exhibited premature germination at the walking-stick-bent cotyledon stages while *abi3* and *aba1* showed a delayed premature germination once embryo growth completed (Figs 5, 7). These results suggest that during embryogenesis there are at least two different pathways for regulation of embryo dormancy. To study the interaction of these two processes, double mutants were constructed between *fus3-8/Ler, lec1-3* or *lec2-1* and *abi3-5* or *aba1-1*. Double mutants between *fus3 abi3* (Keith et al., 1994; Baumlein et al., 1994; Parcy et al., 1997), *lec1 abi3* (Parcy et al., 1997; Meinke et al., 1994), *lec2 abi3* (Meinke et al., 1994), and *fus3 lec1* (West et al., 1994) have been reported previously, but they were never analysed as early as the embryo growth phase.

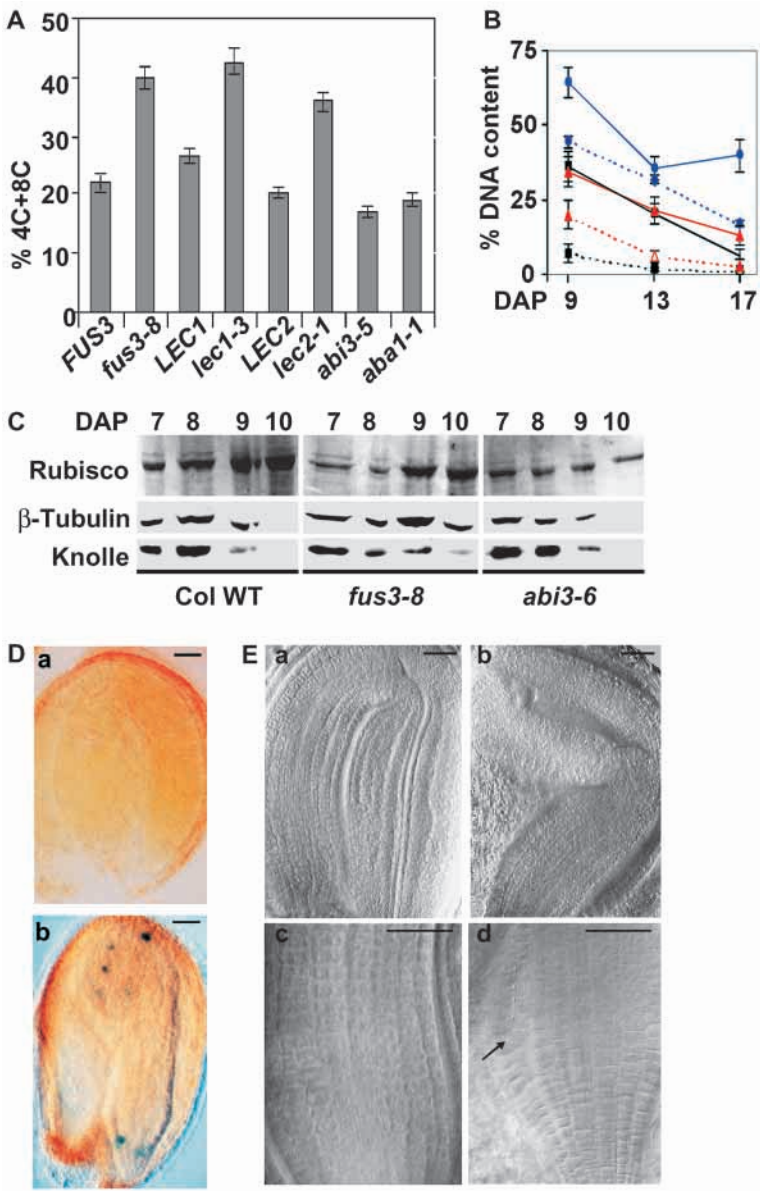


Fig. 3. Cell division in growth arrested mutants. (A) DNA content in seeds 11 DAP. Siliques were harvested from heterozygous *fus3, lec1* and *lec2* plants, and homozygous *abi3* and *aba1* plants, and seeds were sorted according to their phenotype. Relative DNA content of replicating nuclei in the embryo was calculated as described in Methods. (B) DNA contents in *fus3* and *abi3* throughout seed maturation. Seeds were isolated from homozygous *fus3-8* (blue), *abi3-6* (red) and Col wild-type (black) plants 9, 13 and 17 DAP. Solid lines show relative DNA content of replicating nuclei in the embryo; dotted lines show relative DNA content of the endosperm. Averages represent three siliques. (C) Expression of KNOLLE and β-tubulin proteins in mutants. Seeds were isolated, protein were extracted from the seeds and subjected to western blot analysis with anti-KNOLLE antibodies and anti β-tubulin antibodies. Staining of RUBISCO protein was used as a loading control. (D) Cyc1B-GUS expression in mature seeds, 14 DAP. Seeds from *fus3*::Cyc1B-GUS heterozygous transgenic plants were used for GUS staining. A, *FUS3* seed; b, *fus3* seed. (E) Ectopic growth in *fus3* embryo. Embryos at mature stage from heterozygous siliques of *tt3-3* (a,c) and *tt3-3 fus3-8* (b,d) were analysed with Nomarski optics. Arrow indicate extra cell file. Scale bars, 30 μm.

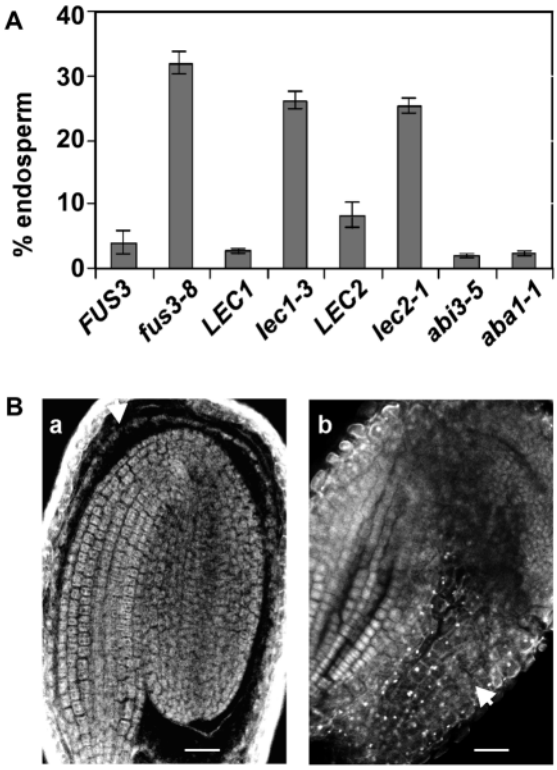


Fig. 4. DNA in the endosperm. (A) DNA content in seeds 11 days after pollination. Siliques were harvested from heterozygous *fus3, lec1* and *lec2* plants and homozygous *abi3* and *aba1* plants. Seeds from heterozygous plants were sorted according to genotype. Embryos were analysed for their DNA content, and the relative DNA content of nuclei in the endosperm (3C+6C) was calculated as described in Methods. (B) Confocal images of mature seeds. Mature seeds, 14 DAP, were harvested from *fus3* heterozygous plants and DNA was stained. A, *FUS3* seed; b, *fus3* seed. Arrowheads indicate endosperm. Scale bars, 30 μm.

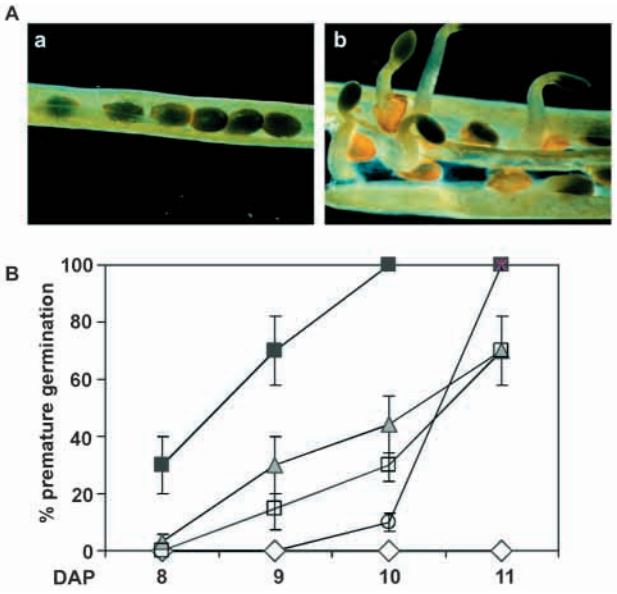


Fig. 5. Premature germination. (A) Siliques of (a) a wild type and (b) a mutant exhibiting premature germination, 4 days after harvesting and plating on a water-agar. (B) Premature germination profile in mutants. Siliques 8-11 DAP were harvested from L*er* wild type (white diamonds); *fus3-8* (black squares); *lec1-3* (grey triangles); *lec2-1* (white squares) and *abi3-5* (white circles) plants, cut open and placed on water-agar plates. Germination was scored after 7 days. Averages represent 60 seeds. Premature germination experiment was repeated at least 3 times.

Premature germination was observed in all double mutants between *fus3, lec1, lec2* and *aba1*or *abi3* (Fig. 7). Double mutants between *embryo growth arrest* mutants and *aba1*exhibited a high percentage of premature germination as early as the walking-stick stage, suggesting additivity. Double mutants with *abi3* showed premature germination from the bent-cotyledon stage; indicating additivity for at least *lec1* and *lec2*, while *fus3 abi3* premature germination was similar to the *fus3* profile of premature germination (Fig. 7). When seeds remained attached to the silique, double mutants *fus3-8/Ler aba1-1* exhibited vivipary within detached green siliques while the parents did not show a vivipary phenotype (Fig. 8A). The double mutants *lec1-3 aba1-1*, and *lec2-1 aba1-1* were also viviparous (not shown). Vivipary was observed when detached siliques were still green and the funiculus was intact, thus the seed was still attached to the maternal tissue. Premature germination, however, is observed in siliques where the seeds had no vascular connection with the mother plant. In the *fus3-8 abi3-5* double mutant, premature germination inside the silique was observed only when siliques started to senesce (Fig. 8A). The same was observed for the double mutants *lec1-3 abi3-5* and *lec2-1 abi3-5* (not shown). Growth of seedlings on the mother plants was further observed when plants were grown under high humidity (90%; not shown), thus the environmental conditions affect the strength of the vivipary phenotype. All double mutants produced trichomes, but compared with the parents they exhibited altered seedling morphology. In combination with *abi3-5*, *fus3-8*, *lec1-3* or *lec2-1* double mutants exhibited fast growth of the seedling (Fig. 7). However, when combined with *aba1-1*, seedlings had wider and shorter hypocotyls (Fig. 7), which at higher magnification appear to result from aberrant cell elongation.

Double mutants *fus3-8 lec1-3*, and *fus3-8 lec2-1*, exhibited a similar premature germination profile as *fus3-8* (Fig. 7A). This epistatic relationship between these three mutants seemed to imply that all three mutant loci regulate the same pathway. However, these results do not exclude the possibility that the mutations have effects at different times of development, since the *embryo growth* phenotype of the *fus3* mutant is expressed earlier (Fig. 2) and can dominate the speed of premature germination in the double mutant. The *abi3-5 aba1-1* double mutant did not germinate prematurely at the walking-stick or bent cotyledon stages. Similar to the parent *abi3-5* at 10-11 DAP and later this double mutant fully germinated (not shown).

These genetic analyses show that at least two pathways regulate premature germination; the combination of both pathways result in premature germination in the silique. Reciprocal crosses between heterozygous *fus3-8 aba1-1* and *aba1-1* revealed that the vivipary phenotype was dependent on maternal *aba1* (Fig. 8B). These results further indicate that a viviparous phenotype in *Arabidopsis* requires mutations in two pathways and the involvement of both maternal and zygotic tissue.

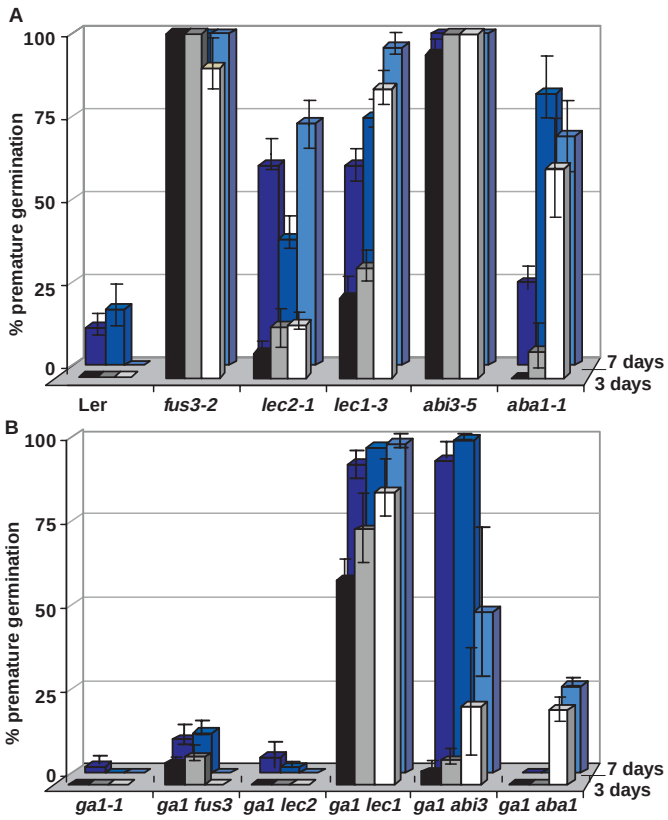


Fig. 6. Germination of single and double mutant seeds excised from siliques at later stages of seed development. Germination of the monogenic mutants (A) or the *ga1-3* double mutants (B) excised at 12 (black and purple bars), 14 (grey and cyan bars), and 18 (white and lilac) DAP and incubated on water-agar plates. Germination was scored after 3 (black to white range) and 7 (blue range) days. Averages are based on germination percentages obtained from four different plants.

Ectopic cell division during postembryonic growth

Recent studies suggest that both *LEC1* and *ABI3* gene function is not restricted to embryogenesis and seed development (Lotan et al., 1998; Rohde et al., 2000). When growing on MS plates for 10 days, prematurely germinated *fus3-8* seedlings exhibited ectopic cell growth of the petiole and cotyledons (Fig. 9Ab,Bc), which was not observed in wild-type seedlings growing in the same conditions. Concentrating on the vegetative area, ectopic expression of Cyc1B-GUS was observed in petioles and in cotyledons of *fus3-8* (Fig. 9Bb,c), while in Col seedlings Cyc1B-GUS was restricted to the shoot apical meristem in seedlings at the same age (Fig. 9Ba). These results indicate that the *FUS3* gene functions during seedling development, possibly to reduce or regulate cell division.

Adventitious shoots that developed on seedlings were observed in double mutants *fus3-8/Ler abi3-5* (Fig. 9Ac). In *fus3-8/Ler lec1-3* double mutants ectopic growth was observed in various parts of the hypocotyls and cotyledons, some of these ectopic structures looked like embryos (Fig. 9Ad), while others resembled shoot-like structures. Additivity between *lec1-3 lec1-2* double mutants during early adult growth also resulted in ectopic shoot growth (Fig. 9Ae). These ectopic growths developed after premature germination. In the double mutant between *fus3-8/Ler lec2-1*, however, ectopic growth was not observed (not shown), which further supports the genetic model in which *fus3* and *lec2* act in the same pathway, while *lec1* regulates a different developmental pathway.

DISCUSSION

In the embryo morphogenesis ends with the completely formed structure, while during seed maturation processes both the embryo and the seed coat are prepared for dormancy. The phase sandwiched between morphogenesis and seed maturation comprises mainly growth processes, which were

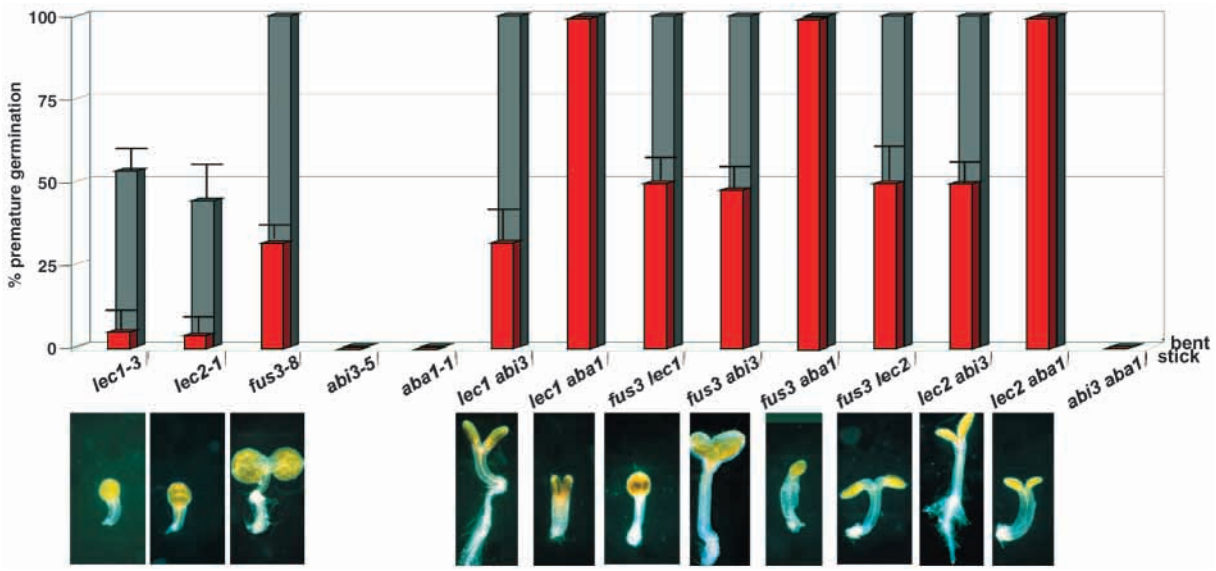
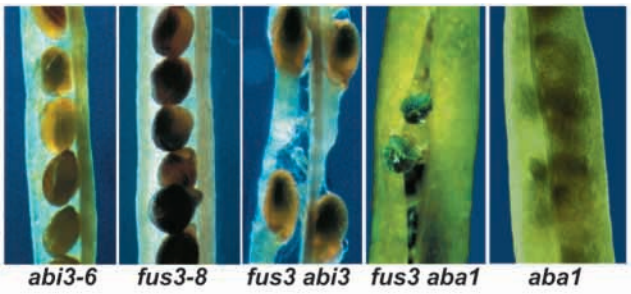


Fig. 7. Premature germination in double mutants. Siliques were harvested at 8 and 9 DAP, from homozygous single and double mutants, cut open and plated on water-agar. Embryo stage was determined at the time of plating, and germination from both walking-stick (red bars) and bent cotyledons (grey bars) stage was scored after 4 days. Averages represent three independent repetitions. Photographs were taken 3 days after plating.

A



B

	Genotypes	Phenotypes
Parents	*f/f a/a*	*vp*
	F/f a/a	WT
	f/f A/a	*pmg*
	♀ ♂	
Crosses: 1	*F/f a/a* × *f/f a/a*	*vp, pmg*
2	*F/f a/a* × *f/f A/a*	*vp, pmg*
3	*f/f A/a* × *F/F a/a*	WT, *pmg*
4	*F/f a/a* × *F/F a/a*	WT
5	*F/f a/a* × *F/f a/a*	WT, *vp, pmg*

Fig. 8. Vivipary in double mutants. (A) Seeds were harvested 16 days after pollination and immediately photographed. Carpals from *abi3-6; fus3-8* and *abi3-6 fus3-8* were removed. (B) The effect of maternal ABA. Table summarises crosses and the resulting phenotypes in *fus3-8 (f), aba1-5 (a)* plants. The viviparous *(vp)* phenotype was determined as in Fig. 8A, premature germination *(pmg)* was determined as in Fig. 6, and WT indicates lack of germination. Siliques were harvested 12-18 DAP. Seeds were plated on water-agar and germination was scored after 3 days.

poorly studied before. The embryo growth phase starts at the torpedo stage and ends with a mature embryo that fills the seed sac. During this phase, which takes 3-4 days in *Arabidopsis*, the ratio between the embryo volume and the endosperm volume is reversed. While at the end of the growth phase the volume of the embryo is increased about tenfold the endosperm is degraded and its volume is reduced to one cell layer. These growth activities are characterised by dramatic changes in the profile of cell division in the embryo and the endosperm. An increase in cell divisions is observed at the beginning of the growth phase, and then is arrested by the end of this phase. Cell division in the embryo resumes after germination. The existence of interactions between the endosperm and the embryo during maize embryogenesis has been suggested (Opsahl-Ferstad et al., 1997; Berger, 1999). Our measurements of DNA content in the embryo and endosperm in the *growth arrest* mutants may indicate such interaction occurs between embryo and endosperm during the embryo growth phase in *Arabidopsis*.

It is of interest to consider if cell division arrest is passively or actively regulated? Mutants, in which the embryo fails to undergo growth arrest show no arrest of cell division in either the embryo or endosperm. The *lec/fus3*-type of embryos do not fill the seed sac, and upon desiccation the seeds show a shrivelled phenotype. Our results indicate that the control of cell division plays an active role during embryo growth arrest and this arrest of cell division activity is possibly regulated by the transcription factors LEC1, FUS3 and the product of *LEC2*. This regulation, which may start during embryo growth phase, correlates with the expression pattern of both *LEC1* (Lotan et al., 1998) and *FUS3* (Luerssen et al., 1998). *FUS3* regulation of cell division may not be restricted to seed development, as

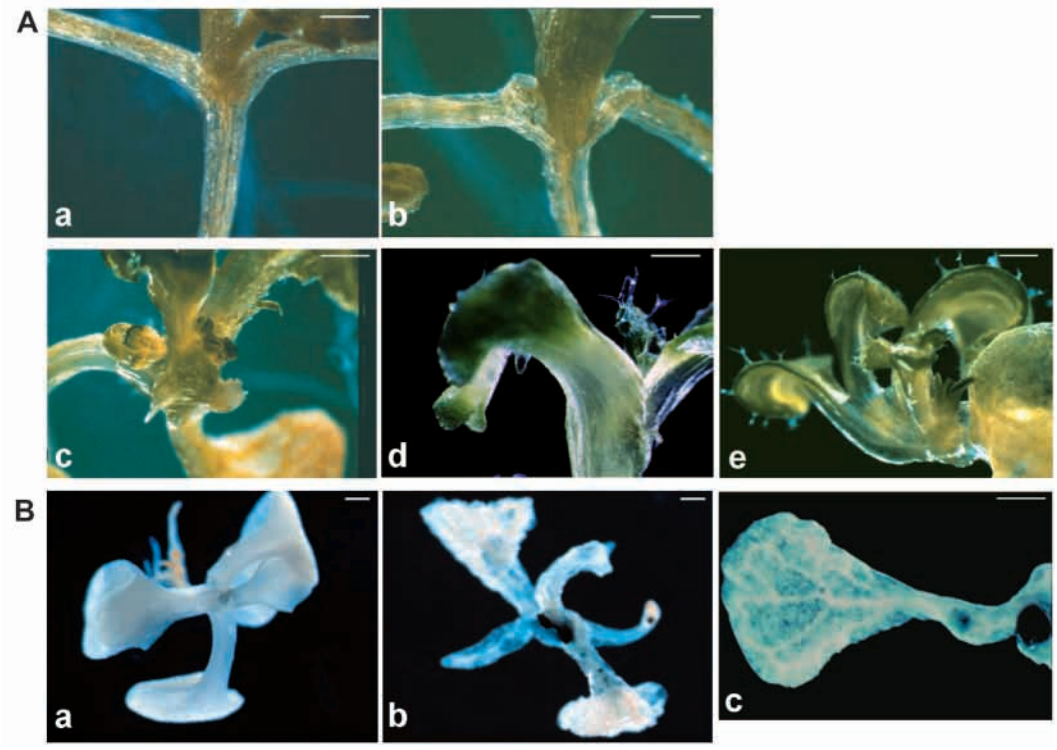


Fig. 9. Postembryonic growth in double mutants. (A) Sterilized seed were plated on MS plates and grown for 10 days. Genotypes: a, Ler wild type; b, *fus3-8*; c, *fus3-8 abi3-5;* d, *fus3-8 lec1-3*; e, *lec1-3 lec2-1*. (B) 10-day- old seedlings of Col::Cyc1B-GUS (a) or *fus3-8*::Cyc1B-GUS (b,c) stained with GUS substrates. Scale bars, 10 mm.

ectopic expression of Cyc1B-GUS was found in *fus3* seedlings. Based on northern blot analyses *LEC1* and *FUS3* mRNA expression was mainly detected during seed development (Lotan et al., 1998; Luerssen et al., 1998), however, the ectopic expression of CycB1-GUS in *fus3* seedlings may indicate low level expression in premature germinated seedlings.

The *abi3* and *aba1* mutants exhibited premature germination when immature seeds are excised from siliques, but unlike *fus3*, *lec1* and *lec2* embryos, which have the potential to grow when excised from the seed at the walking-stick and bent cotyledon stages onwards, the *abi3* and *aba1* embryos failed to grow. At this time normal arrest of cell division in *abi3* and *aba1* mutants was observed, which further supports the idea that these mutants are not defective in embryo growth arrest. The premature germination observed in the latter mutants, together with normal DNA activity in the developing seed implicates the absence of embryo dormancy. These results together indicate that the arrest of embryo growth is secured by two different processes: first arrest of embryo growth, in which cell division is arrested, and later, once embryogenesis is completed, embryo dormancy is signalled (Fig. 10). Premature germination coincides with the early expression of germination genes, first in *fus3* and later in *abi3* (Nambara et al., 2000). Our double mutant analyses further support this model as double mutants exhibited additivity and enhanced premature germination or even vivipary. Double mutant between *fus3-3* and *aba2-2* showed precocious germination, which indicated additivity (Nambara et al., 2000). Vivipary in maize occurs in ABA-deficient and ABA insensitive mutants (Robertson, 1955). In *Arabidopsis*, however, mutations in the homologous genes do not result in vivipary. Here we show that in *Arabidopsis*, vivipary requires two processes, one that is regulated by the *FUS/LEC* genes and a second that requires ABA.

Additivity between *lec1/lec2* mutants and *abi3* has been studied previously (reviewed by Holdsworth et al., 1999; and Wobus and Weber, 1999). In previously reported studies the phenotype of these mutants was not tested as early as the embryo growth phase, since these mutants were considered as seed maturation mutants based on the phenotype of the desiccated seed. Based on premature germination, *fus3* is epistatic to *abi3*. This observation is in agreement with the expression of germination genes in *fus3 abi3* double mutants as early as in the monogenic *fus3* mutant (Nambara et al., 2000). Later during development additivity of *fus3* and *abi3* was observed in the seedling phenotype. Previously reported double mutants of *lec1 abi3* showed germination in the silique while *lec2 abi3* and *fus3 abi3* showed no germination in the siliques (Meinke et al., 1994; Baumlein et al., 1994; Keith et al., 1994). Unlike previous studies we were careful to construct double mutants in the same genetic background. In our system the double mutant *fus3 abi3* exhibited germination in the silique in both Col and L*er* backgrounds. The importance of the genetic background is further shown by the premature germination and germination phenotypes of *lec2-1* in the L*er* background. This mutant in the Ws-1 background did not show a germination phenotype (Meinke et al., 1994).

Additional support for the sequential model for the development of arrest in seeds (see Fig. 10), comes from the genetic analyses of the *fus3-8 aba1-5* double mutant. We show that the ABA component is maternal, thus the additivity does not come only from two developmentally distinguishable steps,

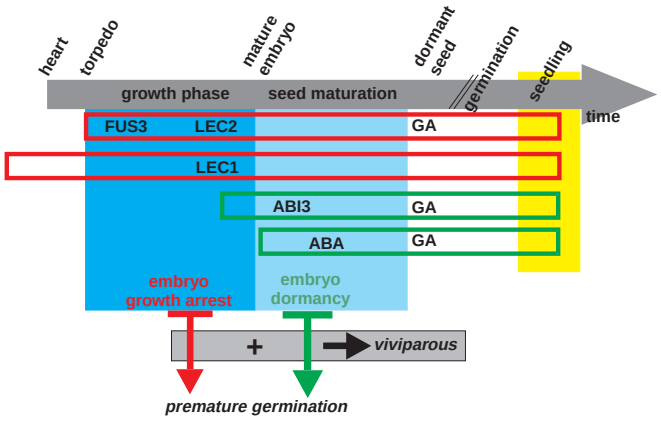


Fig. 10. A schematic diagram for sequential model of developmental arrest in seeds. During the growth phase the embryo expression of *FUS3*, *LEC1* and *LEC2* result in growth arrest. Mutations in these genes result in reduced embryo growth arrest. Early during the seed maturation phase activity of *ABI3* and *ABA1* cause embryo dormancy and mutations in these genes result in premature germination. Premature germination is regulated by GA-dependent and GA-independent pathways. Vivipary and premature germination inside the silique occurs in *embryo growth arrest* and *embryo dormancy* double mutants. Red and green boxes indicate gene expression and function.

but also from two different tissue origins. Maternal ABA in the seed was reported before (Karssen et al., 1983) but shown to be less relevant for dormancy than embryonic ABA at later stage of seed development and in mature seeds. The results presented here indicate that the maternal ABA component in the seed can inhibit viviparous germination in mutants that lack the growth arrest after the embryo phase.

Premature germination is also dependent on the germination hormone gibberellin. When combined *growth arrest* mutants, except *lec1*, with the *ga1* mutant the double mutants failed to exhibit premature germination. This confirms the observation of Keith et al. (1994) that germination of the *fus3-3* mutant can be inhibited by the GA biosynthesis inhibitor paclobutrazol. *lec1*, however, did show a GA-independent premature germination. These results indicate that *FUS3* and *LEC2* are involved in a regulatory pathway, which is different from the *LEC1* pathway (Fig. 10). Our analyses of seedling growth further support our model that *FUS3* and *LEC2* are involved in the same pathway, as additivity was not observed. *LEC1* and *FUS3* were suggested to regulate different pathways based on lack of epistasis (West et al., 1994). However, while we could not determine epistasis between *lec1* and *fus3* based on premature germination, our studies on postembryonic growth indicated additivity between the two mutants. Double mutants between *fus3* and *lec1* were suspected of additivity as they formed a higher proportions of twin embryos (Lotan et al., 1998). In our system, however we rarely observed twin embryos (1-2%, not shown).

Embryo growth arrest is a transient phase, the embryo is signalled to arrest its growth during the embryo growth phase and continue its growth upon germination. Thus, study of such growth processes cannot be done after the process is complete. The analyses of transient growth processes require detailed time-dependent analyses during growth (Raz and Ecker, 1999), and will benefit from a reversible induction system in planta.

As shown in this study analysing mutants that were previously characterized as affecting later developmental processes, can reveal more details about the regulation of the embryo growth phase when analysed in time-dependent experiments.

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