

RESEARCH PAPER

LEC1*, *FUS3*, *ABI3* and *Em* expression reveals no correlation with dormancy in *Arabidopsis

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Abstract

Dormant *Arabidopsis* seeds require stratification and light for germination. To study gene expression during establishment, maintenance and release of dormancy, various *Arabidopsis* ecotypes that are different in their degree of dormancy were investigated; three *nsm* mutants that lack the stratification-dependency, and the precocious germination and reduced dormancy of the *abi3-1* mutant (insensitive to ABA). Genes examined by mRNA abundance include *LEC1*, *FUS3* and *ABI3*, transcription factors that are major regulators of embryo development and, at least indirectly, play some role in the control of dormancy. Moreover, the late embryogenesis marker genes, *AtEm1* and *AtEm6*, were examined in relation to the state of dormancy. The expression of *LEC1*, *FUS3* and *ABI3* mRNA is only marginally different during seed development in various strong or moderate dormancy wild types, *nsm* mutants and *abi3-1*. Therefore, it is unlikely that these transcription factors directly control the establishment of dormancy in *Arabidopsis*. Sole and various combinations of light, temperature, and after-ripening regimes that alter germination behaviour were examined to determine if the expression of *ABI3*, *AtEm1* and *AtEm6* mRNAs were correlated with dormancy-breaking processes. *ABI3* expression is influenced by cold and light, in a similar way in both dormant and non-dormant wild-type seeds. *ABI3* transcript abundance in the *nsm1* and *nsm2* mutants is higher and in the *nsm5-1* mutant is marginally lower than in wild-type seeds, but changes due to temperature and light factors are very similar to those that occur in wild-type

seeds. The abundances of *AtEm1* and *AtEm6* mRNAs are equally affected by imbibition and cold temperature in mature and after-ripened seeds. The *LEA* transcript abundances for *AtEm1* and *AtEm6* are reduced in *nsm* mutants in a common, *ABI3*-independent pathway.

Key words: *ABI3*, *Arabidopsis thaliana*, *Em* genes, *FUS3*, gene expression, germination, late embryogenesis, *LEC1*, mRNA, seed development, seed dormancy.

Introduction

The embryos of many seed plants have the remarkable property that they are able to go into a period of dormancy, which is characterized by the inability to germinate immediately in otherwise supportive conditions until specific environmental stimuli break or relieve this inhibition of germination. Despite many genetic and molecular studies of factors involved in embryogenesis and seed development (see reviews by Bewley, 1997; Galau *et al.*, 1991; Goldberg *et al.*, 1989; Hilhorst and Toorop, 1997; Kermode, 1990; Li and Foley, 1997; McCarty, 1995), the major genes controlling mature seed dormancy exclusively have not been identified.

Germination of fresh *Arabidopsis* seeds requires light and a cold treatment (stratification), which is an imbibition at 4 °C for several days. Dormancy is not broken by cold treatment of dry *Arabidopsis* seed alone; the cold-signal response requires that the seed be hydrated. Extensive dry storage results in seed ageing (after-ripening), after which germination occurs in less restrictive imbibition conditions (Hilhorst and Karssen, 1992). Various *Arabidopsis* eco-

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types can be differentiated in their stratification and initial primary dormancy grade. *INSOMNIAC* (*NSM*) mutant alleles, lacking the stratification-breakable component of mature seed dormancy, have been isolated in *Arabidopsis* (Baumbusch, 2001). The mature seeds of *nsm* mutants are inhibited by exogenous abscisic acid, they retain the normal light-breakable component of dormancy and the plants have normal vegetative and reproductive growth (Baumbusch, 2001). *Arabidopsis* mutants with reduced dormancy (*rd*), but otherwise wild-type behaviour, have been described by Léon-Kloosterziel *et al.* (1996), but these mutants show a large variation in the mutant and wild-type phenotypes. The sequences and characteristics of the *RDO* genes are still unknown.

The *viviparous-1* (*vp-1*) mutant in maize (McCarty *et al.*, 1989; Neill *et al.*, 1987) and *abscisic acid-insensitive* (*abi*) mutants in *Arabidopsis* (Finkelstein, 1994; Koornneef *et al.*, 1984) are capable of germinating in the presence of normally inhibitory concentrations of ABA. The *Arabidopsis abi* mutants have severe alterations including reduced dormancy (Finkelstein, 1994; Koornneef *et al.*, 1984; Nambara *et al.*, 1994, 1992; Ooms *et al.*, 1993; Parcy *et al.*, 1994). The studies of *vp1* and *abi3* mutants, focusing on late embryo functions such as desiccation tolerance, have not yet determined why the mutants are so non-dormant, but it is suggested that ABA is involved (Nambara *et al.*, 1992; Ooms *et al.*, 1993). ABA is probably involved in initiating late embryogenesis processes, but it is unlikely to be the exclusive major trigger for controlling the different aspects of seed development and dormancy (Bewley, 1997; Galau *et al.*, 1991; Giraudat *et al.*, 1994; Rock and Quatrano, 1995).

A number of mutants have been isolated with disturbed embryonic development programmes including perturbed dormancy establishment. For example, *leafy cotyledon* (*lec*) mutants of *Arabidopsis* are unable to distinguish between embryonic and vegetative patterns of plant development. *Lec* mutations exhibit morphological characteristics such as altered cotyledon morphology, desiccation intolerance and occasional vivipary (Meinke, 1992; West *et al.*, 1994). The *LEC1* gene encodes a transcription factor exclusively accumulating during seed development (Lotan *et al.*, 1998). The *fusca3* (*fus3*) mutation of *Arabidopsis* affects several aspects of embryogenesis, provoking a *lec*-like phenotype with ectopic trichomes, desiccation intolerance and precocious germination (Bäumlein *et al.*, 1994; Keith *et al.*, 1994; Nambara *et al.*, 2000). The *FUS3* and the *ABI3* gene encode transcription factors that are similar to those encoded by the *VP1* gene (Giraudat *et al.*, 1992; Luerksen *et al.*, 1998; McCarty *et al.*, 1991). *LEC1*, *FUS3* and *ABI3* are postulated to play an important role in controlling mid- to late embryogenesis (Bäumlein *et al.*, 1994; Castle and Meinke, 1994; Keith *et al.*, 1994; Nambara *et al.*, 2000; Vicent *et al.*, 2000; West *et al.*, 1994) and the establishment and maintenance

of dormancy (Parcy *et al.*, 1997). Studies of the wild type and *abi3 fus3* double mutants suggest a negatively regulating function for *ABI3* and *FUS3* genes during late embryogenesis, possibly the inhibition of a particular set of genes during late embryo development (Nambara *et al.*, 2000).

LEA (*Late Embryogenesis-Abundant*) genes are expressed late in embryogenesis (Galau *et al.*, 1987, 1991; Hughes and Galau, 1991). *LEA* gene expression has been used as a convenient marker for altered expression in dormancy mutants or in mutations which affect dormancy. In *Arabidopsis*, the *Em*-like class I *LEA* genes, *AtEm1* and *AtEm6*, have been cloned and characterized (Finkelstein, 1993; Gaubier *et al.*, 1993). The *fus3* and the *lec1* mutants show altered expression of *AtEm1* and *AtEm6* mRNAs (Vicent *et al.*, 2000).

An analysis of the establishment, maintenance and breaking of dormancy at the physiological and gene expression level using known gene expression markers is reported here. In order to elucidate genes supposed to be involved in the establishment of dormancy, the abundance of *LEC1*, *FUS3* and *ABI3* mRNAs in various *Arabidopsis* ecotypes during silique development was determined. Furthermore, gene expression changes of *ABI3* and the *LEA* genes, *AtEm1* and *AtEm6*, in *nsm* mutants and the *Ws* wild type during variable conditions have been studied to investigate the impact of temperature, light and after-ripening time on the maintenance and breaking of dormancy.

Materials and methods

Plant material

The *Ws* (Wassilewskija) ecotype of *Arabidopsis thaliana* was obtained from David Meinke (Department of Botany, Oklahoma State University, Stillwater, OK). The ecotype *Cvi* was obtained from the *Arabidopsis* Biological Resource Center at Ohio State University (Columbus, OH). Ecotype *C24* and mutant line *abi3-1* (in *Ler* background) were obtained from T Palva (Helsinki, Finland). Seeds containing the *ABA-insensitive3* (*abi3-1*) mutant allele, in *Ler* background, are generally considered to be non-dormant (Koornneef *et al.*, 1989; Ooms *et al.*, 1993).

The *nsm* mutants *nsm1*, *nsm2* and *nsm5-1* were isolated on the basis of their rapid germination in light, prior to stratification, and their phenotypes operationally defined as *INSOMNIAC* (Baumbusch, 2001). They are in *Ws* background and those used here were backcrossed at least once to *Ws*. The *nsm5* mutants are hypersensitive to ABA, but appear normal in all other aspects of plant growth and development. Four *nsm5* mutant alleles have been described and two additional mutants *nsm1* and *nsm2* have been characterized (Baumbusch, 2001). Complementation and physical mapping studies show that the *nsm1*, *nsm2* and *nsm5* mutants are in different genes at separate positions.

Growth and harvesting conditions

Plants were routinely grown in environmentally-controlled growth rooms (22 °C, 8/16 h dark/light, 100 $\mu\text{E m}^{-2} \text{s}^{-1}$). For germination experiments, the plants were grown under continuous cool-white fluorescent light (50 $\mu\text{E m}^{-2} \text{s}^{-1}$) at 20 \pm 2 °C. For developmental

experiments, staging of developing siliques was performed by tagging individual flowers on the day of pollination, which was defined as the day the petals were visible. Only flowers borne on primary inflorescences were used and siliques were collected during 0–18 days after pollination (dap) of development. Under growth chamber conditions the seeds reached the mature stage at 15–16 dap. For each stage, a pool of siliques was harvested from 24 plants (0–5 dap), 12 plants (6–15 dap) and 24 plants (15–18 dap). Siliques were harvested and immediately frozen in liquid nitrogen (Parcy *et al.*, 1994).

Germination conditions

After incubation treatments, the pooled seeds were collected in a tube and immediately frozen in liquid nitrogen and stored at -80°C .

The seeds were sown on $1\times$ MS inorganic medium (Murashige and Skoog, 1962) containing 2.5 mM 2-*N*-morpholinoethanesulphonic acid (MES), adjusted to pH 5.7 by the addition of KOH. Phytigel (Sigma) was added to 0.45% (w/v) as a solidifying agent prior to autoclaving. The autoclaved medium was poured into polystyrene plates. Seeds were sprinkled onto plates without prior sterilization. For incubation at room temperature and under light, the seeds were placed under standard germination conditions with growth room light standards (22°C , 8/16 dark/light, $100\ \mu\text{E m}^{-2}\ \text{s}^{-1}$). The plates of sown seeds were scored for germination daily by indicating on the bottom of the plate beneath each seed the day on which germination occurred. After 6 d, the plate was removed to 4°C for 4 d to stratify non-germinated seeds, and the plates were scored for germination upon return to 22°C . For incubation at a low temperature under light, the seeds were placed in a cold room at 4°C under standard light conditions (8/16 h dark/light, $100\ \mu\text{E m}^{-2}\ \text{s}^{-1}$). For incubations in the dark, the plates containing the seeds were wrapped under complete darkness in two layers of aluminium foil and placed in a light-proof box within a few seconds after sowing. The wrapped plates were transferred to the appropriate temperature conditions at 22°C or 4°C , respectively. Standard stratification, when done, was at 4°C for 4 d.

For after-ripening experiments, mature seeds of the *Arabidopsis* wild-type ecotypes Ws, C24 and Cvi were placed on phytigel plates at weekly intervals and germination was measured over a period of 4 weeks.

Following germination testing, the seeds were stratified for up to three cycles to examine the stratification requirements for germination and the viability of the seed.

For stratification and after-ripening experiments, seeds were staged as follows. (a) Mature seed: fresh mature seeds, dark-brown and completely dry; siliques dry and yellow-brown. Seeds reached this stage after about 15–16 d post-pollination. The seeds were mature, but not after-ripened. (b) Mature seeds after-ripened for 4 weeks: mature seeds were removed from siliques and stored for 4 weeks in air at room temperature and under room light. Mature seeds were taken from eight plants and pooled together as a single sample and a minimum of 30–50 μg seeds for mature (0 weeks) or 4-week samples was aliquotted from the same pool of seeds. After each treatment, the seeds were frozen in liquid nitrogen and kept at -80°C . The treatments for seeds of both ages included no incubation, incubation at 4°C or 22°C , and incubation in dark or light as described above.

RNA analyses

RNA was extracted using a modified protocol described by Downing *et al.* (1992). Approximately 0.1–1 g fresh weight of tissue, frozen in liquid N_2 , was transferred to a glass homogenization unit containing 1 ml lysis buffer (100 mM TRIS-HCl, pH 8.0, 500 mM LiCl, 10 mM EDTA, pH 8.0, 1% LiDS, 1 mM DTT). The tissue was allowed to thaw during the 0.5–2.5 min homogenization. 900 μl phenol-

chloroform-isoamyl alcohol (25:24:1 by vol.) was added to 900 μl of the extract. The solution was mixed for 30 s by vortex and incubated for 5–15 min on ice. After centrifugation at 11 500 rpm, for 15 min (this and all the following centrifugations were performed at 4°C), the upper (aqueous) layer was collected and a second extraction with phenol-chloroform-isoamyl alcohol (25:24:1 by vol.) was performed followed by a final extraction with 1 vol. chloroform. For total RNA precipitation, one-third vol. 8 M LiCl was added to 2 M LiCl and incubated on ice overnight. After centrifugation at 9000 rpm, for 30 min, the RNA pellet was resuspended in 100 μl TE (10 mM TRIS-HCl, 1 mM EDTA, pH 8). For the second RNA precipitation, 150 μl 5M KAc, pH 6, was added and left for 3–5 h on ice. RNA was collected by centrifugation at 9000 rpm for 30 min and redissolved in 100 μl TE. For the ethanol precipitation, 200 μl ETOH and 10 μl 3 M NaAc, pH 5.2, were added and the mixture incubated at -20°C for 30–120 min. After centrifugation at 12 000 rpm for 30 min, the pellet was washed once with 70% EtOH and centrifuged for 5 min at 12 000 rpm. The drained pellet was dried under vacuum. RNA was allowed to dissolve for 5 min at 60°C in 30 μl TE with periodic vortexing. RNA concentration was estimated by absorbance at 260 nm of a suitably diluted 2 μl aliquot.

Total RNA (10 μg) was size-fractionated on a 1.7% agarose, 3% formaldehyde gel (Sambrook *et al.*, 1989), transferred to nylon filters (Hybond-N, Amersham) by capillary action and afterwards UV-cross linked with 120 000 mJ cm^{-2} (Hoefer system). Filters were hybridized at 68°C with *AtEm1*, *AtEm6*, *LEC1*, *FUS3*, and *ABI3* probes according to the method of Church and Gilbert (1984) and at 70°C with a rDNA probe to verify that equal amounts of RNA were present in each line. Filters were stripped with 1 mM EDTA, pH 8.0, 0.1% SDS for subsequent rehybridization. Autoradiographic signals were scanned and quantified. ^{32}P -labelled DNA probes from PCR reactions were generated in a random-priming reaction with biotinylated single-stranded template bound to magnetic streptavidin-coated beads (Dynabeads M280-streptavidin, Dynal Biotech AS, Norway and GenoPrep™ Streptavidin beads, Genovision, Norway) as described (Espelund *et al.*, 1990). The *AtEm1*, *AtEm6*, *LEC1*, *FUS3*, and *ABI3* probes were generated in a standard random-priming reaction using a Random Primed DNA labelling kit (Roche, Switzerland). *AtEm1* (454 bp) primers: *AtEm1*-f 5'-GTCCGTA-TCTGTGTCGGAAC-3' and *AtEm1*-r 5'-CACTGCCGTTAGTAC-AAGCC-3'; *AtEm6* (279 bp) primers *AtEm6*-f 5'-CGTATGAC-GTACCGATTGTC-3' and *AtEm6*-r 5'-GGTCATCACCATCC-LEC1 (626 bp) primers *LEC1*-f 5'-GTATCGTGGTCCAGCAGC-AA-3' and *LEC1*-r 5'-GGATTCATCTTGACCCGACG-3'; *FUS3* (927 bp) primers *FUS3*-f 5'-CTCATGGTCTGCAGCTAGGTG-3' and *FUS3*-r 5'-CGAATGTTCCGAATTGGAG-3'; *ABI3* (2162 bp) primers *ABI3*-f 5'-GGATGGAATTGATGGAAGTTG-3' and *ABI3*-r 5'-GCATGTCTCCACCACTGTTA-3'. The rDNA probe was generated using a biotinylated single-stranded template bound to streptavidin-coated beads in a specific priming reaction (Stacy *et al.*, 1991), rRNA (1803bp) NS3-F 5'-GCAAGTCGTGTGCC-AGCAGCC-3' and NS4-r 5'-CTTCCGTCAATTCCTTTAAG-3'.

Results

Degree of dormancy in wild-type ecotypes

The Ws ecotype exhibits only moderate seed dormancy. Therefore, the germination of other *Arabidopsis* ecotypes was examined over an after-ripening period of 4 weeks to identify ecotypes having higher levels of dormancy. The results of this survey are reported in Table 1. The *Arabidopsis* ecotypes, Cvi, C24 and Ws, are completely dormant as fresh mature seeds. The germination of seeds of

Table 1. Percentage germination in relations to after-ripening time and stratification cycles of the ecotypes Ws, Cvi and C24

After-ripening time is given in weeks, the number of repeated stratification treatments is given as 1st, 2nd and 3rd, not stratified seeds are marked with not stratified and post-stratification germination is indicated with post-stratification. Values are the means of \pm SD of percentage germination at 4 d of 3–5 replicates. Seed incubation was at room temperature under light (for details see Materials and methods).

Ecotype	Weeks after-ripening											
	0			1			2			4		
	Not stratified	Post-stratification		Not stratified	Post-stratification		Not stratified	Post-stratification		Not stratified	Post-stratification	
Ws	1 \pm 2	100		19 \pm 15	100		60 \pm 30	100		7 \pm 4	100	
			1st stratification		1st stratification			1st stratification			1st stratification	
			2nd stratification		2nd stratification			2nd stratification			2nd stratification	
			3rd stratification									
C24	0	8 \pm 6		0	80 \pm 10		0	100		2 \pm 2	100	
Cvi	0	0		0	5 \pm 3		0	97 \pm 6		0	100	
			1st stratification		1st stratification			1st stratification			1st stratification	
			2nd stratification		2nd stratification			2nd stratification			2nd stratification	
			3rd stratification									

the Ws ecotype increased during the 4-week test period to a final germination of 97% after 4 weeks of after-ripening. By contrast, Cvi and C24 seeds remain dormant even after 4 weeks of after-ripening. As another indicator of dormancy strength, germination was assayed after stratification treatments. The number of stratification treatments required to completely break dormancy in mature seeds is ecotype-dependent. For fresh seeds of the Ws ecotype, a single immediate stratification is sufficient to break dormancy completely, resulting in a 100% germination within 4 d upon return to room temperature conditions (Table 1). Thus, the Ws wild type can be classified as moderately dormant. By contrast, fresh mature seeds of Cvi required two cycles of stratification punctuated by 2 d at room temperature before dormancy was broken. On the basis of both the time requirement for after-ripening and the immediate stratification demand of mature seeds, the three ecotypes tested can be rated as Ws << C24 < Cvi, from moderate to highly dormant.

Expression of *LEC1*, *FUS3* and *ABI3* mRNAs during silique development

Investigations of developmental differences in the accumulation kinetics of *LEC1*, *FUS3* and *ABI3* mRNAs of the ecotypes Cvi, C24, Ws and the *nsm1*, *nsm2*, *nsm5-1*, and *abi3-1* mutants were performed using developmental northern blots of total silique RNA (Fig. 1). All three mRNAs have been found to be expressed only in embryos (Bäumlein *et al.*, 1994; Giraudat *et al.*, 1992; Lotan *et al.*, 1998; Nambara *et al.*, 2000, 1995; West *et al.*, 1994). Each probe detected a single band, as expected. Hybridization with gene specific probes was followed by hybridization with an 18S rDNA probe to detect any RNA loading differences. No major differences in RNA loading were noted (Figs 2, 3; for Fig. 1 data not shown). *LEC1* transcript expression was detected at low intensity throughout silique development. The different ecotypes, Cvi, C24 and Ws, show the highest *LEC1* mRNA expression at 4–7 dap (Fig. 1). Compared with the Ws wild type, the *nsm1* accumulates transcript a day earlier, beginning at 3 dap. By contrast, *nsm5-1* has high transcript abundance for at least a day longer than the other ecotypes and mutants, from 3–8 dap. The *abi3-1* mutant shows weaker accumulation through at least 5 dap, with a maximal expression at 7 dap (Fig. 1). *FUS3* transcripts accumulate later during silique development. In Cvi, C24 and Ws *FUS3* transcript increases at 8 dap, lasting until 14 dap for Cvi and C24 and until 13 dap for Ws. All the mutants begin to accumulate *FUS3* at a slightly earlier time. The *nsm1* and *nsm2* mutants begin to accumulate *FUS3* 2–3 d earlier, and *nsm5-1* and *abi3-1* about 1 d earlier than the wild types. High *FUS3* transcript expression is terminated by 14 dap in all ecotypes and mutants (Fig. 1). Detectable *ABI3* mRNA expression starts at about 5 dap, with a pattern of increasing abundance during

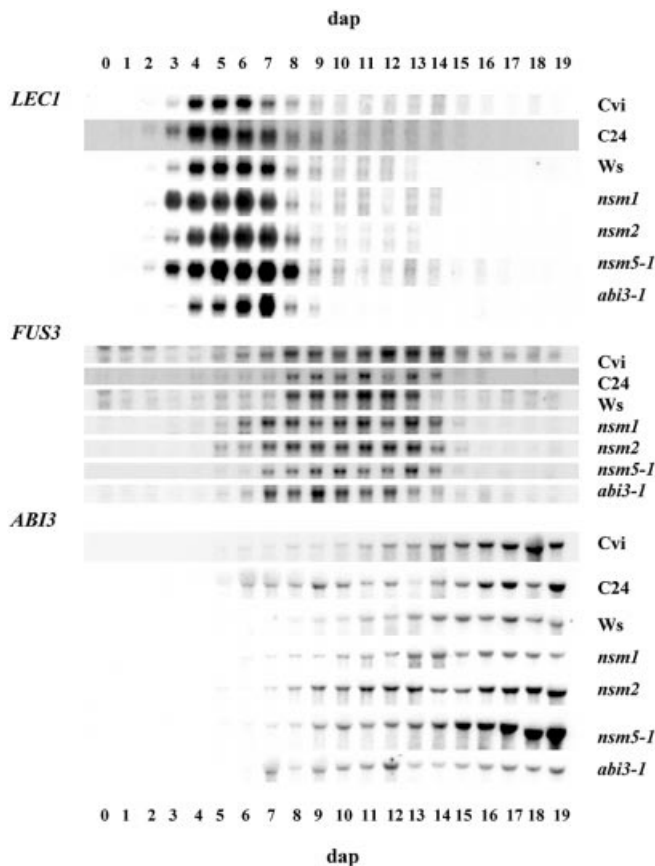


Fig. 1. Comparative gel blot analysis of the expression patterns of *LEC1*, *FUS3* and *ABI3* mRNAs during silique development of the Cvi, C24, Ws ecotypes, and the *nsm1*, *nsm2*, *nsm5-1*, and *abi3-1* mutants. The probes were hybridized to 10 μ g of total RNA isolated from siliques 0–17 dap (for *LEC1*) and 0–19 dap (for *FUS3* and *ABI3*). Mature seed stage was reached at 15–16 dap (days after pollination). Equal loading was controlled by hybridization of blots with 18S rDNA probes.

silique development with a maximal accumulation at 19 dap. All the wild types and the *nsm1*, *nsm2* and *nsm5-1* and *abi3-1* mutants show a similar abundance of the *ABI3* transcripts, however, *nsm5-1* may accumulate transcript at higher concentrations through 19 dap than the others (Fig. 1). Given the potential uncertainties in the age of the siliques used for RNA preparations, it was concluded that differences in the rate of development are insignificant among genotypes, and that no obvious correlation between developmental expression and dormancy degree of the mature seeds emerges.

AtEm1, *AtEm6* and *ABI3* gene expression in mature and after-ripened seeds

Total RNA from the ecotypes Cvi, C24, Ws, the *nsm*-mutants, *nsm1*, *nsm2*, *nsm5-1*, and the *abi3-1* mutant was extracted from the mature seeds and from the mature seeds that had been after-ripened for 4 weeks. For the wild types and mutants, only marginal transcript concentration

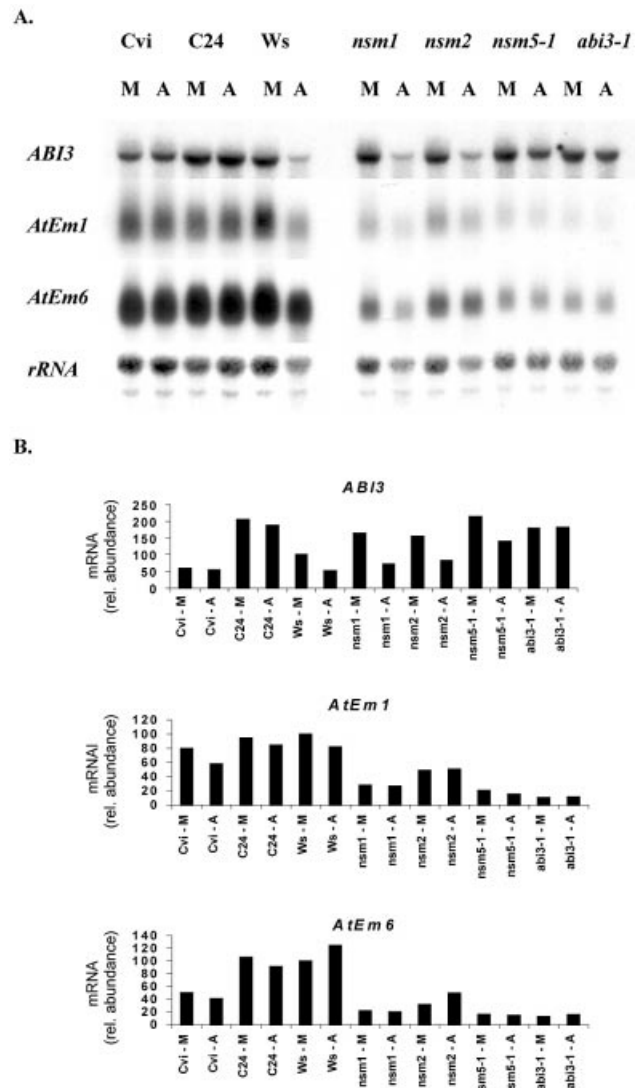


Fig. 2. Northern blot analysis showing mRNA abundance of *ABI3* and *LEA* genes *AtEm1* and *AtEm6* in mature and after-ripened seeds of the Cvi, C24, Ws ecotypes, and the *nsm1*, *nsm2*, *nsm5-1*, and *abi3-1* mutants. (A) Blots (the same blots were used for all hybridization probes). (B) Estimated abundance from quantitation of hybridization signals. M, mature seeds; A, mature seeds aged 4 weeks. Relative abundances of the transcripts in the histogram displayed were corrected for variation in loading by comparison with rRNA amounts in each lane.

differences are detectable between mature and after-ripened seeds. All *nsm* mutants and the *abi3-1* mutant show about half of the *AtEm1* and *AtEm6* transcript accumulation compared with the Ws wild type (Fig. 2). The C24 and Ws ecotypes display similar *AtEm1* and *AtEm6* transcript amounts, with a 2-fold higher abundance than the Cvi ecotype (Fig. 2). C24 seeds show greater *ABI3* content than the other ecotypes, 2-fold higher than Ws and 4-fold higher than Cvi (Fig. 2). All the *nsm* mutants have, as mature or after-ripened seeds, a 1.5–2-fold higher *ABI3*

expression quantity than the Ws wild type. Both the Ws wild type and all *nsm* mutants have half the *ABI3* transcript amount in after-ripened seeds compared with the mature seeds. The *abi3-1* mutant shows a high *ABI3* accumulation at mature and after-ripened stage (Fig. 2). The results of the gene expression studies reported in Fig. 2 indicate that the differences in *AtEm1*, *AtEm6* and *ABI3* abundance of the various ecotypes and mutants are not related to differences in dormancy levels.

Influence of light, temperature and after-ripening time on AtEm1, AtEm6 and ABI3

Dormant wild-type *Arabidopsis* seeds require a combination of imbibition, temporally defined cold stimulus (stratification) and light to break dormancy. *LEA* genes might be involved in the inhibition pathway of germination during the later stages of development. Transcript accumulations of *AtEm1*, *AtEm6* and *ABI3* were investigated under variable physiological conditions in order to investigate a possible functional relationship between *LEA* genes and seed dormancy. Mature Ws seeds remain dormant after a 4 d incubation at room temperature in light or darkness, but after-ripened Ws seeds germinate immediately (Table 1; data not shown). Mature seed dormancy in the Ws ecotype is broken by a cold incubation at 4 °C for 4 d (Table 1). All *nsm* mutants germinate to 100% without prior stratification (Baumbusch, 2001). Despite the lack of stratification dependency, the light requirement for germination in *nsm* mutants is still present.

Thus, *nsm* mutants are only affected in the stratification-breakable, but not in the light-perceptible, part of seed dormancy (Baumbusch, 2001).

After-ripening, humidity, temperature, and light influence the state of dormancy in mature Ws wild-type seeds (Table 2). The influence of these factors on transcript abundances were determined in Ws wild-type seeds and compared with non-dormant *nsm1*, *nsm2* and *nsm5-1* mutants using mRNAs probes corresponding to *AtEM1*, *AtEm6* and *ABI3* (Fig. 3). Equal loading was tested with ribosomal RNA detection using radioactive 18S rDNA probe.

Mature and dormant Ws wild-type seeds (M1) have about one-third lower *AtEm1* and *AtEm6* transcript concentrations than the mature seeds imbibed at 22 °C in darkness (M2; see Fig. 3). The stratification treatment of mature seeds incubated for 4 d at low temperature (4 °C) in darkness (M3) or light (M4) results in a dramatic reduction in *AtEm1* and *AtEm6* expression compared with mature seeds (M1) or with mature seeds which have been incubated at 22 °C in the dark (M2). Remarkably, an equal reduction in *LEA* gene transcripts is visible in after-ripened seeds. After-ripened non-dormant (A1) seeds have about one-third lower *AtEm1* and *AtEm6* transcript amounts than in after-ripened seeds imbibed at 22 °C in darkness (A2). Moreover, after-ripened seeds, incubated at 4 °C in dark (A3) or light (A4), have less than a quarter *AtEm1* and *AtEm6* mRNA accumulation than that observed in after-ripened seeds (A1) or in after-ripened

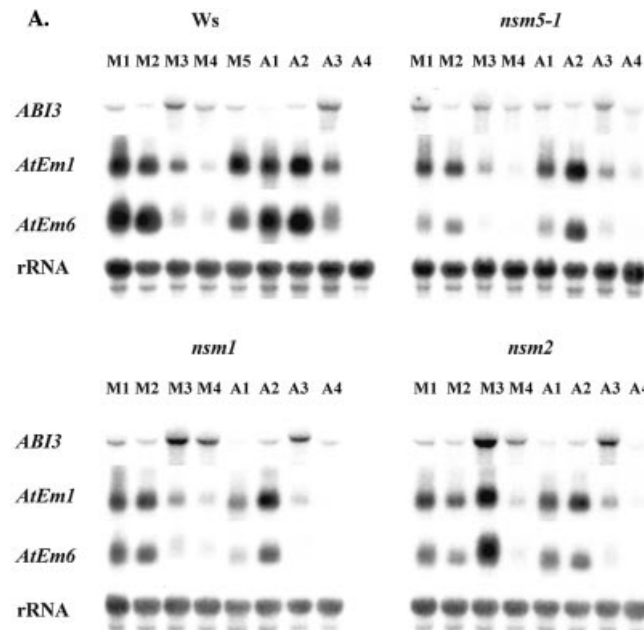


Fig. 3. (A) RNA blot analysis of mRNA abundance of *ABI3* and the *LEA* genes *AtEm1* and *AtEm6* in mature seeds under various temperatures, times and light conditions. Each lane contained 10 µg total RNA from freshly harvested mature seeds (M1, 2, etc) or 4 week after-ripened seeds (A1, 2, etc). Seeds were either directly frozen in liquid nitrogen (0 d) or incubated (4 d) at 22 °C or 4 °C in darkness or under 8/16 h dark/light (for detailed information about various conditions and predicted germination behaviour see Table 2).

seeds which have been imbibed at 22 °C in darkness (A2). For both cases of mature and after-ripened wild-type seeds, *LEA* gene transcript abundances are substantially lower after 4 °C incubation in light (M4 or A4) compared with seeds incubated at 4 °C in darkness (M3 or A3). These results indicate that light has a modulating effect on *LEA* gene transcription abundance in *Ws* wild-type seeds (Fig. 3).

In mature (M1), after-ripened (A1) or in mature and after-ripened seeds which have been incubated at 22 °C in darkness (M2 and A2), *AtEm1* accumulations in *Ws* wild-type seeds are equal to or higher than in all *nsm* mutants. Under similar conditions *AtEm6* accumulations in the wild type are 2–7-fold higher than in all *nsm* mutants. These higher abundances occur in both mature (M1) and mature

incubated at 22 °C in darkness seeds (M2), as well as in after-ripened (A1) and after-ripened incubated at 22 °C in darkness seeds (A2). Remarkably, a strong increase in transcript abundance in mature *nsm2* seeds is seen at 4 °C cold incubation in darkness (M3), 7-fold for *AtEm1* and 14-fold for *AtEm6*, but not during cold incubation in light (M4). In summary, wild-type seeds have a lower *LEA* gene expression during cold temperature incubation, but equally for mature (dormant) and for after-ripened (non-dormant) seeds. The *nsm* mutants, compared with the wild type, generally have decreased *LEA* mRNA accumulation.

ABI3 mRNA accumulation in mature wild-type seeds incubated at 4 °C in darkness (M3) is 10-fold higher compared with the expression in mature seeds (M1) as well as in seeds which have been incubated at 22 °C in darkness

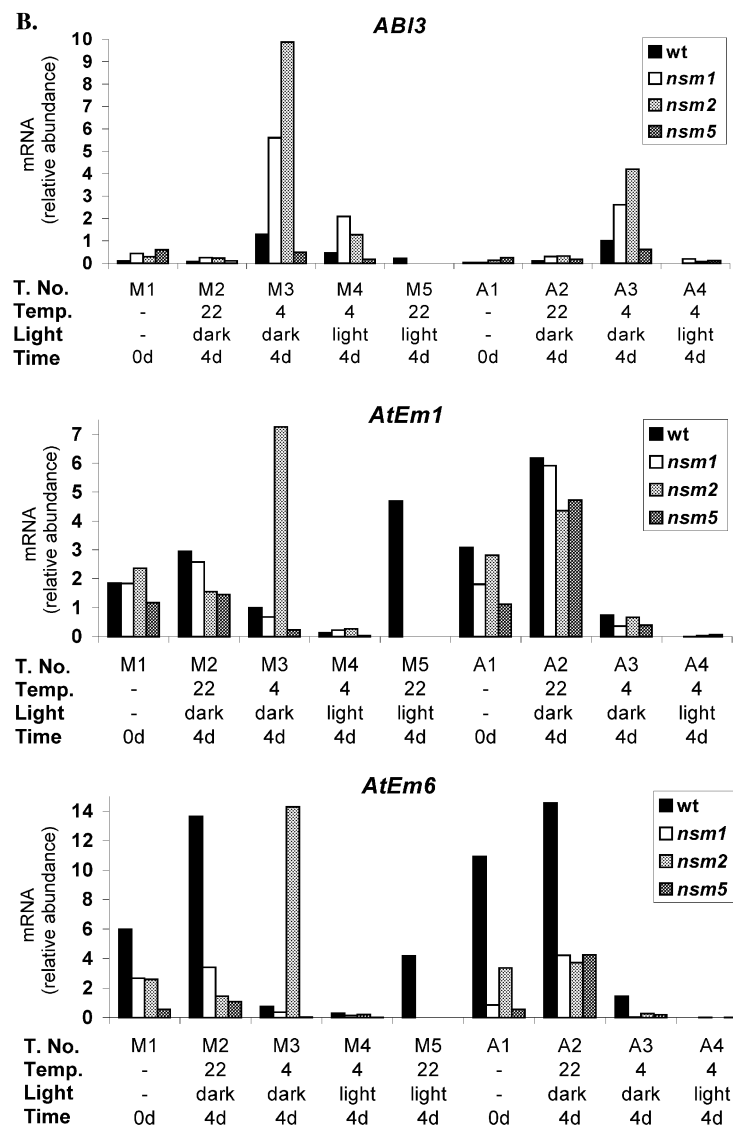


Fig. 3. (B) Relative abundance of transcripts, measured as the intensity of the hybridization signals and normalized for loading. Different treatment numbers (T. No.) correspond with conditions in Table 2.

Table 2. *Physiological conditions in relation to amount of germination for gene expression studies with different nsm mutants*

Ws wild type and the *nsm1*, *nsm2* and *nsm5-1* mutants lines were used for incubation experiments under various temperatures, times and light conditions. Freshly harvested mature seeds (M) and 4 week after-ripened seeds (A) were either directly frozen in liquid nitrogen (0 d) or incubated (4 d) at 22 °C or 4 °C in darkness (dark) or under 8/16 h dark/light (light). All seeds were intact at the time of RNA extraction. The predicted germination behaviour is indicated as percentage germination; superscript D, dormant; superscript ND, non-dormant; superscript NG, non-germinating.

Treatment stage	No.	Genotype	Conditions			Predicated germination behaviour	
			Temperature	Light	Time	At 4 d	At 4 d+2 d
Mature	M1	Ws	–	–	0 d	0% ^D	0% ^D
		<i>nsm1</i>	–	–	0 d	100% ND	100% ND
		<i>nsm2</i>	–	–	0 d	100% ND	100% ND
		<i>nsm5-1</i>	–	–	0 d	100% ND	100% ND
	M2	Ws	22 °C	Dark	4 d	0% ^D	0% ^D
		<i>nsm1</i>	22 °C	Dark	4 d	100% ND	100% ^{NG}
		<i>nsm2</i>	22 °C	Dark	4 d	100% ND	100% ^{NG}
		<i>nsm5-1</i>	22 °C	Dark	4 d	100% ND	100% ^{NG}
	M3	Ws	4 °C	Dark	4 d	0% ^D	100% ^{ND, NG}
		<i>nsm1</i>	4 °C	Dark	4 d	100% ND	100% ^{NG}
		<i>nsm2</i>	4 °C	Dark	4 d	100% ND	100% ^{NG}
		<i>nsm5-1</i>	4 °C	Dark	4 d	100% ND	100% ^{NG}
	M4	Ws	4 °C	Light	4 d	0% ^D	100% ^{ND, NG}
		<i>nsm1</i>	4 °C	Light	4 d	100% ND	100% ^{NG}
		<i>nsm2</i>	4 °C	Light	4 d	100% ND	100% ^{NG}
		<i>nsm5-1</i>	4 °C	Light	4 d	100% ND	100% ^{NG}
4 weeks	M5	Ws	22 °C	Light	4 d	0% ^D	0% ^D
		<i>nsm1</i>	–	–	0 d	0% ^D	100% ND
		<i>nsm2</i>	–	–	0 d	100% ND	100% ND
		<i>nsm5-1</i>	–	–	0 d	100% ND	100% ND
	A2	Ws	22 °C	Dark	4 d	100% ND	100% ^{NG}
		<i>nsm1</i>	22 °C	Dark	4 d	100% ND	100% ^{NG}
		<i>nsm2</i>	22 °C	Dark	4 d	100% ND	100% ^{NG}
		<i>nsm5-1</i>	22 °C	Dark	4 d	100% ND	100% ^{NG}
	A3	Ws	4 °C	Dark	4 d	100% ND	100% ^{NG}
		<i>nsm1</i>	4 °C	Dark	4 d	100% ND	100% ^{NG}
		<i>nsm2</i>	4 °C	Dark	4 d	100% ND	100% ^{NG}
		<i>nsm5-1</i>	4 °C	Dark	4 d	100% ND	100% ^{NG}
	A4	Ws	4 °C	Light	4 d	100% ND	100% ^{NG}
		<i>nsm1</i>	4 °C	Light	4 d	100% ND	100% ^{NG}
		<i>nsm2</i>	4 °C	Light	4 d	100% ND	100% ^{NG}
		<i>nsm5-1</i>	4 °C	Light	4 d	100% ND	100% ^{NG}

(M2). Similarly, after-ripened (A3) wild-type seeds incubated at 4 °C in darkness show an approximately 10-fold enhanced *ABI3* mRNA accumulation than the after-ripened seeds (A1) or after-ripened seeds incubated at 22 °C in darkness (A2). As was observed for the *LEA* gene expression, the presence of light modifies the temperature dependence of transcript concentrations. Mature wild-type seeds incubated at 4 °C in darkness (M3) have twice the *ABI3* abundance of 4 °C plus light-incubated seeds (M4) and four times the *ABI3* abundance of seeds which have been incubated in 22 °C plus light (M5). Compared with the wild-type, *ABI3* transcript concentrations in *nsm1* and *nsm2* mutants are higher in mature or after-ripened seeds at 4 °C incubation in darkness or light (M3, A3 and M4).

The results show that *ABI3* gene expression is induced equally by cold temperature incubation in darkness and in mature and after-ripened seeds of wild-type genotypes. This induction is even stronger in *nsm1* and *nsm2*. Thus,

there are no clear dormancy-related differences in expression of *ABI3*.

Discussion

Characterizations of *Arabidopsis* plants from different geographical regions and ecosystems suggest that there is considerable genetic variation (reviewed in Alonso-Blanco and Koornneef, 2000). Early observations of seed germination established that there were differences in stratification dependence, after-ripening time, and storage conditions among different *Arabidopsis* ecotypes (Kugler, 1951; Laibach, 1951; Langridge, 1957). The *Arabidopsis* ecotypes, Cvi, C24 and Ws, which were not included in these early studies, were tested. C24 and Cvi are completely dormant as mature and after-ripened seeds and Ws seeds are dormant as mature seeds, but germinate 100% after 4 weeks of after-ripening. Based on their

germination frequency and stratification requirements, the tested ecotypes can be ranked from moderately to strongly dormant, i.e. Ws << C24 < Cvi. This ranking complements a recent investigation of Ws versus the *Ler*, *Col* and *En* ecotypes (Debeaujon *et al.*, 2000). *Cvi* is a strongly dormant ecotype and therefore has been used for creating recombinant inbred lines derived from crosses with the low-dormancy *Ler* ecotype for analysis of quantitative trait loci affecting dormancy (Alonso-Blanco *et al.*, 2003; van der Schaar *et al.*, 1997).

The interaction of three physiological factors, age of seed, incubation of imbibed seed at low temperature (of an ecotype-specific duration), and the correct quality and quantity of light, is necessary for breaking dormancy in wild-type *Arabidopsis*. No single one of these factors breaks dormancy, but after-ripening reduces the requirement for stratification (Table 1). The expression of the well-characterized regulatory genes, *LEC1*, *FUS3*, and *ABI3*, and the post-abscission-abundant *LEA* genes, *AtEm1* and *AtEm6*, were investigated. Transcript abundance was investigated in the moderately dormant Ws ecotype versus the strongly dormant *Cvi* and *Col* ecotypes, as well as in *nsm* mutants lacking the stratification requirement for germination.

Under no conditions could dormancy-dependent gene expression differences between strongly dormant and moderately dormant ecotypes be detected in mature or after-ripened seeds. Seeds of the *aba-1* and *abi3-4* mutants are reported to have reduced *AtEm1* and *AtEm6* accumulation (Parcy *et al.*, 1994) and mutant seeds of *abi3-1* have reduced *AtEm6* accumulation (Finkelstein, 1993). It has been shown here that the *AtEm1* accumulation of *abi3-1* mutant seeds is also reduced. The developmental mutants *lec1* and *fus3* show similar altered *LEA* gene expression (Vicent *et al.*, 2000). The expression in *lec1-1* and *lec1-2* in mature seeds at different stages of development has been described as being reduced for *AtEm6* for both mutants compared with the wild-type and being unchanged for *AtEm1* (Vicent *et al.*, 2000). The results presented here show that all *nsm* mutants have lowered *AtEm1* and *AtEm6* expression in mature and after-ripened seeds compared with the wild type with the exception of an extremely high induction by stratification of *AtEm1* and *AtEm6* in *nsm2* with respect to the other mutants and wild types. The reduced *LEA* abundance in *nsm* mutants could be explained by lower than normal transcription. Consequently, since three different *nsm* mutants show a similar reduction in *LEA* transcripts concentration, the lesions must affect different components in the regulatory pathway of at least the *LEA* genes. This might reflect that *nsm* mutants are skipping a developmental stage that involves preparation for dormancy. However, this preparation cannot involve desiccation protection, because *nsm* dry seeds are viable. In these experiments, the amount of *LEA* transcripts are increased by imbibition and reduced by

cold temperature, with a light-induced modulation effect. Their induction during imbibition could to be a hydration effect or just preparation for dormancy, since it is an open question if dormancy is imposed upon imbibition rather than after abscission or during drying. The observation of reduced *LEA* mRNA accumulation after a 4-week after-ripening time in *Cvi* and *Col* ecotypes, suggests that *LEA* gene expression in mature seeds is a useful marker for dormancy and it is possible that *LEA* gene expression is associated with dormancy regulation pathways. However, involvement of these *LEA* genes with desiccation protection is unlikely. First, dry seeds of the mutants are viable. Second, *LEA* expression is considerably lessened in the mature seeds of the mutants and declines in the *nsm* mutants upon imbibition.

The three genes *LEC1*, *FUS3* and *ABI3* have been found to be general regulators for different aspects of seed development between mid to late embryogenesis (Bäumlein *et al.*, 1994; Parcy *et al.*, 1997; West *et al.*, 1994) including dormancy (Parcy *et al.*, 1997). *LEC1* mRNA accumulation starts at preglobular to bent cotyledon stage, is limited to seed development, and is high during seed maturation (Lotan *et al.*, 1998; West *et al.*, 1994). In this study, *LEC1* transcripts were much higher in abundance at 3–7 dap than subsequently at 8–16 dap. With these experimental conditions, *FUS3* expression was observed during mid-embryogenesis and *ABI3* expression was observed in mid- to late embryogenesis for the Ws, C24 and *Cvi* ecotypes (Fig. 1). Earlier investigations of *FUS3* expression for the Dijon-G or *Col* ecotypes (Luerksen *et al.*, 1998) and *ABI3* expression for the *Ler* ecotype (Parcy *et al.*, 1994) coincide with this temporal accumulation.

Different *Arabidopsis* ecotypes show variation in their dormancy behaviour and in their expression of the two *LEA* mRNAs in mature seeds, but not in the temporal mRNAs accumulation during silique development of the transcription factors *LEC1*, *FUS3* and *ABI3* (Fig. 1). This observation suggests that these transcription factors are not the major immediate regulators required for the establishment of dormancy. In *nsm* mutants, which lack stratification-breakable seed dormancy, the abundance of *LEC1*, *FUS3* and *ABI3* mRNAs are not, or only marginally, changed during development compared with the Ws wild type. It therefore seems unlikely that these transcription factors are the cardinal triggers for the regulation of stratification-breakable dormancy in *Arabidopsis*.

The *nsm* mutants show decreased *AtEm1* and *AtEm6* mRNA abundance, but generally increased *ABI3* mRNA abundance compared with the wild type. *ABI3* mRNA abundance in *nsm* seeds is especially high after imbibition in the cold, under conditions that break dormancy in wild-type seeds. However, similar cold-related alterations in *ABI3* mRNA are observed in wild-type mature dormant and after-ripened non-dormant seeds when imbibed. *ABI3*

mRNA accumulations undergo similar after-ripening age-related changes in the *nsm* mutants and the wild type. In *nsm* mutants, the strongly cold-induced *ABI3* gene expression is reduced by light. These results suggest that the *ABI3* gene is regulated by a cold- and a light-modulated mechanism not directly related to the state of dormancy. In addition, the non-dormant *nsm* mutants show the strongest *ABI3* expression during cold incubation. Further investigations will show if cold-induced genes such as the *SFR* genes (Thorlby *et al.*, 1999) are influenced by *NSM* genes or vice versa. The *nsm* mutants have normal vegetative and reproductive growth, yet only marginal changes in the expression of *LEA* genes and embryo-regulating genes so far examined. However, the variety of *ABI3* and *LEA* gene expression across the *nsm* mutants suggests that the particular genes have several distinct functions in a common dormancy-related pathway.

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References

- Alonso-Blanco C, Bentsink L, Hanhart CJ, Blankestijn-de Vries, Koornneef M. 2003. Analysis of natural allelic variation at seed dormancy loci of *Arabidopsis thaliana*. *Genetics* **164**, 711–729.
- Alonso-Blanco C, Koornneef M. 2000. Naturally occurring variation in *Arabidopsis*: an under-exploited resource for plant genetics. *Trends in Plant Science* **5**, 22–29.
- Bäumlein H, Miséra S, Luerssen H, Kolle K, Horstmann C, Wobus U, Müller AJ. 1994. The *FUS3* gene of *Arabidopsis thaliana* is a regulator of gene expression during late embryogenesis. *The Plant Journal* **6**, 379–387.
- Baumbusch LO. 2001. Identification and analysis of genes controlling embryogenesis and embryo dormancy in plants. PhD thesis, University of Oslo, Norway.
- Bewley JD. 1997. Seed germination and dormancy. *The Plant Cell* **9**, 1055–1066.
- Castle LA, Meinke DW. 1994. A *FUSCA* gene of *Arabidopsis* encodes a novel protein essential for plant development. *The Plant Cell* **6**, 25–41.
- Church GM, Gilbert W. 1984. Genomic sequencing. *Proceedings of the National Academy of Sciences, USA* **81**, 1991–1995.
- Debeaujon I, Léon-Kloosterziel KM, Koornneef M. 2000. Influence of the testa on seed dormancy, germination, and longevity in *Arabidopsis*. *Plant Physiology* **122**, 403–414.
- Downing WL, Mauxion F, Fauvarque MO, Reviron MP, de Vienne D, Vartanian N, Giraudat J. 1992. A *Brassica napus* transcript encoding a protein related to the Kunitz protease inhibitor family accumulates upon water stress in leaves, not in seeds. *The Plant Journal* **2**, 685–693.
- Espelund M, Stacy RA, Jakobsen KS. 1990. A simple method for generating single-stranded DNA probes labelled to high activities. *Nucleic Acids Research* **18**, 6157–6158.
- Finkelstein RR. 1993. Abscisic acid-insensitive mutations provide evidence for stage-specific signal pathways regulating expression of an *Arabidopsis* late embryogenesis-abundant (*lea*) gene. *Molecular and General Genetics* **238**, 401–408.
- Finkelstein RR. 1994. Mutations at two new *Arabidopsis* ABA responsive loci are similar to the *abi3* mutations. *The Plant Journal* **5**, 765–771.
- Galau GA, Bijaisoradat N, Hughes DW. 1987. Accumulation kinetics of cotton late embryogenesis-abundant mRNAs and storage protein mRNAs: co-ordinate regulation during embryogenesis and the role of abscisic acid. *Developmental Biology* **123**, 198–212.
- Galau GA, Jakobsen KS, Hughes DW. 1991. The controls of late dicot embryogenesis and early germination. *Physiologia Plantarum* **81**, 280–288.
- Gaubier P, Raynal M, Hull G, Huestis GM, Grellet F, Arenas C, Pages M, Delseny M. 1993. Two different *Em*-like genes are expressed in *Arabidopsis thaliana* seeds during maturation. *Molecular and General Genetics* **238**, 409–418.
- Giraudat J, Hauge Brian M, Valon C, Smalle J, Parcy F, Goodman Howard M. 1992. Isolation of the *Arabidopsis ABI3* gene by positional cloning. *The Plant Cell* **4**, 1251–1261.
- Giraudat J, Parcy F, Bertauche N, Gosti F, Leung J, Morris PC, Bouvier-Durand M, Vartanian N. 1994. Current advances in abscisic acid action and signalling. *Plant Molecular Biology* **26**, 1557–1577.
- Goldberg RB, Barker SJ, Perez-Grau L. 1989. Regulation of gene expression during plant embryogenesis. *Cell* **56**, 149–160.
- Hilhorst HWM, Karssen CM. 1992. Seed dormancy and germination: the role of abscisic acid and gibberellins and the importance of hormone mutants. *Plant Growth Regulation* **11**, 225–238.
- Hilhorst HWM, Toorop PE. 1997. Review on dormancy, germinability, and germination in crop and weed seeds. *Advances in Agronomy* **61**, 111–165.
- Hughes DW, Galau GA. 1991. Developmental and environmental induction of *Lea* and *LeaA* mRNAs and the post-abscission program during embryo culture. *The Plant Cell* **3**, 605–618.
- Keith K, Kraml M, Dengler NG, McCourt P. 1994. *fusca3*: a heterochromatic mutation affecting late embryo development in *Arabidopsis*. *The Plant Cell* **6**, 589–600.
- Kermode AR. 1990. Regulatory mechanisms involved in the transition from seed development to germination. *Critical Reviews in Plant Science* **9**, 155–195.
- Koornneef M, Hanhart CJ, Hilhorst HWM, Karssen CM. 1989. *In vivo* inhibition of seed development and reserve protein accumulation in recombinants of abscisic acid biosynthesis and responsiveness mutants in *Arabidopsis thaliana*. *Plant Physiology* **90**, 463–469.
- Koornneef M, Reuling G, Karssen CM. 1984. The isolation and characterization of abscisic acid-insensitive mutants of *Arabidopsis thaliana*. *Physiologia Plantarum* **61**, 377–383.
- Kugler I. 1951. Untersuchungen über das Keimverhalten einiger Rassen von *Arabidopsis thaliana* (L.) Heynh. Ein Beitrag zum Problem der Lichtkeimung. *Beiträge zur Biologie der Pflanze* **28**, 211–243.
- Laibach F. 1951. Über sommer- und winterannuelle Rassen von *Arabidopsis thaliana* (L.) Heynh. Ein Beitrag zur Ätiologie der Blütenbildung. *Beiträge zur Biologie der Pflanze* **28**, 173–210.
- Langridge J. 1957. The aseptic culture of *Arabidopsis thaliana* (L.) Heynh. *Australian Journal of Biological Sciences* **10**, 243–252.
- Léon-Kloosterziel KM, van de Bunt GA, Zeevaart JA, Koornneef M. 1996. *Arabidopsis* mutants with a reduced seed dormancy. *Plant Physiology* **110**, 233–240.
- Li BL, Foley ME. 1997. Genetic and molecular control of seed dormancy. *Trends in Plant Science* **2**, 384–389.
- Lotan T, Ohto M, Yee KM, West MA, Lo R, Kwong RW,

- Yamagishi K, Fischer RL, Goldberg RB, Harada JJ.** 1998. *Arabidopsis* *LEAFY COTYLEDON1* is sufficient to induce embryo development in vegetative cells. *Cell* **93**, 1195–1205.
- Luerssen H, Kirik V, Herrmann P, Misera S.** 1998. *FUSCA3* encodes a protein with a conserved VP1/ABI3-like B3 domain which is of functional importance for the regulation of seed maturation in *Arabidopsis thaliana*. *The Plant Journal* **15**, 755–764.
- McCarty DR.** 1995. Genetic control and integration of maturation and germination pathways in seed development. *Annual Review of Plant Physiology and Plant Molecular Biology* **46**, 71–93.
- McCarty DR, Carson CB, Stinard PS, Robertson DS.** 1989. Molecular analysis of *viviparous-1*: an abscisic acid-insensitive mutant of maize. *The Plant Cell* **1**, 523–532.
- McCarty DR, Hattori T, Carson CB, Vasil V, Lazar M, Vasil IK.** 1991. The *viviparous-1* developmental gene of maize encodes a novel transcriptional activator. *Cell* **66**, 895–905.
- Meinke DW.** 1992. A homeotic mutant of *Arabidopsis thaliana* with leafy cotyledons. *Science* **258**, 1647–1650.
- Murashige T, Skoog F.** 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473–497.
- Nambara E, Hayama R, Tsuchiya Y, Nishimura M, Kawaide H, Kamiya Y, Naito S.** 2000. The role of *ABI3* and *FUS3* loci in *Arabidopsis thaliana* on phase transition from late embryo development to germination. *Developmental Biology* **220**, 412–423.
- Nambara E, Keith K, McCourt P, Naito S.** 1994. Isolation of an internal deletion mutant of the *Arabidopsis thaliana* *ABI3* gene. *Plant Cell Physiology* **35**, 509–513.
- Nambara E, Keith K, McCourt P, Naito S.** 1995. A regulatory role for the *ABI3* gene in the establishment of embryo maturation in *Arabidopsis thaliana*. *Development* **121**, 629–636.
- Nambara E, Naito S, McCourt P.** 1992. A mutant of *Arabidopsis* which is defective in seed development and storage protein accumulation is a new *abi3* allele. *The Plant Journal* **2**, 435–441.
- Neill SJ, Horgan R, Rees AF.** 1987. Seed development and vivipary in *Zea mays* L. *Planta* **171**, 358–364.
- Ooms JJJ, Léon-Kloosterziel K, Bartels D, Koornneef M, Karssen CM.** 1993. Acquisition of desiccation tolerance and longevity in seeds of *Arabidopsis thaliana*. A comparative-study using abscisic acid-insensitive *abi3* mutants. *Plant Physiology* **102**, 1185–1191.
- Parcy F, Valon C, Kohara A, Misera S, Giraudat J.** 1997. The *ABSCISIC ACID-INSENSITIVE3*, *FUSCA3*, and *LEAFY COTYLEDON1* loci act in concert to control multiple aspects of *Arabidopsis* seed development. *The Plant Cell* **9**, 1265–1277.
- Parcy F, Valon C, Raynal M, Gaubier-Comella P, Delseny M, Giraudat J.** 1994. Regulation of gene expression programs during *Arabidopsis* seed development: roles of the *ABI3* locus and of endogenous abscisic acid. *The Plant Cell* **6**, 1567–1582.
- Rock CD, Quatrano RS.** 1995. The role of hormones during seed development. In: Davies PJ, ed. *Plant hormones*. Dordrecht, The Netherlands: Kluwer Academic Publishers, 671–697.
- Sambrook J, Fritsch EF, Maniatis T.** 1989. *Molecular cloning: a laboratory manual*. New York: Cold Spring Harbor Laboratory Press.
- Stacy JE, Ims RA, Stenseth NC, Jakobsen KS.** 1991. Fingerprinting of diverse species with DNA probes generated from immobilized single-stranded DNA templates. *Nucleic Acids Research* **19**, 4004.
- Thorlby G, Veale E, Butcher K, Warren G.** 1999. Map positions of *SFR* genes in relation to other freezing-related genes of *Arabidopsis thaliana*. *The Plant Journal* **17**, 445–452.
- van der Schaar W, Alonso-Blanco C, Léon-Kloosterziel KM, Jansen RC, van Ooijen JW, Koornneef M.** 1997. QTL analysis of seed dormancy in *Arabidopsis* using recombinant inbred lines and MQM mapping. *Heredity* **79**, 190–200.
- Vicient CM, Bies-Etheve N, Delseny M.** 2000. Changes in gene expression in the *leafy cotyledon1* (*lec1*) and *fusca3* (*fus3*) mutants of *Arabidopsis thaliana* L. *Journal of Experimental Botany* **51**, 995–1003.
- West MA, Yee KM, Danao J, Zimmermann JL, Fischer RL, Goldberg RB, Harada JJ.** 1994. *LEAFY COTYLEDON1* is an essential regulator of late embryogenesis and cotyledon identity in *Arabidopsis*. *The Plant Cell* **6**, 1731–1745.