Molecular and genetic mechanisms regulating the transition from embryo development to germination

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How do plants regulate development from embryogenesis through to germination? Mutational analysis of embryo maturation and germination in Arabidopsis and maize has provided a framework for dissecting the regulatory processes required for this transition. Genetic loci have been identified that are responsible for both repressing premature germination and simultaneously stimulating embryo development. Several of these loci have now been cloned, and an analysis of their molecular properties, in combination with analysis of their genetic interactions, is providing insights into how the transition between seed and seedling is regulated and coordinated.

One of the first scientific experiments that children often do is to plant a seed and watch it germinate. The reanimation of the seemingly dead seed is fascinating to see, and has probably set more than a few plant scientists on the path to their future careers. Of course, this apparently simple phenomenon is no such thing and results from highly complex and regulated sets of developmental processes designed to maximize the chances of survival for the seedling. Typically, towards the end of embryo development, storage products are deposited that are subsequently broken down and used by the germinating seedling as an initial food source. Desiccation tolerance increases as water is removed from the drying seed, and in most flowering plants embryo development and germination are separated by a period of quiescence, during which the embryo remains in a desiccated state as part of the dry seed. Quiescence can occur at one of several stages of embryo development, or not at all (for example, mangroves continuously develop from embryo to seedling). In addition to a quiescent phase, many mature seeds also show a period of dormancy, where the embryo does not germinate under otherwise favourable conditions. Only after a period of ‘after-ripening’ (during which the capacity for dormancy is lost by the dry seed) will they then germinate. Obviously, the catabolic processes that occur before germination, and the catastrophic processes that occur afterwards are mutually exclusive, and it is important for the development of the seedling that they are separated developmentally. Of course, this apparently simple phenomenon is probably set more than a few plant scientists on the path to their future careers. Of course, this apparently simple phenomenon is probably set more than a few plant scientists on the path to their future careers. Of course, this apparently simple phenomenon is probably set more than a few plant scientists on the path to their future careers.

Genetic screens have revealed many loci that control embryo maturation and germination. These fall broadly into two groups, the majority having an influence over many stages of plant growth and development, with a small number whose effects are seed specific (Fig. 1). The largest class of loci is that encoding proteins regulating hormone production or sensitivity. The controlling influence of plant hormones on the transition between embryo and seedling was established many years ago. Abscisic acid (ABA) has been shown to be a potent inhibitor of germination in many species, whereas gibberellic acid (GA) promotes germination. Genetic evidence that ABA enhances seed maturation and represses germination has been provided by the identification of loci encoding proteins involved in ABA synthesis and perception. Mutations at these loci disrupt embryo maturation (including the accumulation of storage reserves), desiccation tolerance and embryo dormancy. The aba mutants of Arabidopsis and several viviparous (vp; precocious germination of embryos) mutants of maize contain low levels of ABA, and these loci encode enzymes involved in ABA biosynthesis. The abscisic acid insensitive (abi) mutants (and vp 1 in maize), have normal levels of ABA, but are hypo-sensitive to applied ABA (Refs 2,5,6), and mutations at the Arabidopsis ERA1 locus exhibit an enhanced response to ABA, indicating a role for these loci in ABA signal transduction. The phenotypes of several of these loci, as revealed by mutant analysis, are specific to the seed, for example, abi 1, abi 4 and abi 5 (Fig. 1).

Gibberellic acid has a strong positive effect on germination. Mutations that decrease levels of active GA, or sensitivity to GA, also influence germination potential. Studies have indicated that in Arabidopsis both GA biosynthesis and perception are required for germination. Therefore, antagonism between remaining ABA levels in the dry seed, and GA levels and perception inhibitors might contribute to the developmental decision between dormancy or germination.

Several other loci have been identified that specifically affect seed maturation and germination, which do not appear to be directly related to hormone synthesis or signalling. These are the leafy cotyledon (LEC) class genes, including LEC1, LEC2 and FUSC3 (FUS3; Fig. 2) 8, 9. Mutations at these loci characteristically result in the production of trichomes on cotyledons (these epidermal hairs are normally absent from cotyledons but present on leaves) and anthocyanin production, which causes a purple colouration at the tips of the cotyledons. Both loci, and fusc3 mutant embryos can also show vivipary and are desiccation-intolerant, unlike loc2 mutants. All can be ‘rescued’ and produce normal adult plants, indicating that their effects are specific to embryo
development. Mutant lec 1 seeds show an extreme alteration in morphology, including rounded cotyledons, constricted roots and a shortened axis (Fig. 2), whereas fus 3 embryos appear morphologically more normal but a small proportion of seeds produce double embryos. Cotyledon identity is altered in both fus 3 and lec 1 mutant embryos, cells appear to be intermediate in structure between typical cotyledon and leaf cells, and storage protein accumulation in the embryos is greatly reduced.

The phenotypes of lec 1 and fus 3 seeds are similar in several ways to abi 3 seeds, including the lack of desiccation tolerance, and the shrunken appearance of cotyledons. Most interestingly, with respect to the transition from embryo to seedling, all show seedling characteristics during embryo development, including premature activation of the shoot apical meristem (Fig. 2) and significant vascular differentiation. This suggests that post-germination characteristics are expressed in mutant embryos, and that these loci repress the expression of post-germination characteristics during embryo development.

Several different studies have analysed the genetic relationships between ABI 3, LEC 1 and FUS 3 by observing phenotypes of double mutant seeds. These phenotypes indicate that these loci regulate both overlapping and separate biochemical and developmental pathways. Integration of their functions prevents premature expression of germination characteristics and promotes characteristics associated with embryo development. The combination of abi 3 with either lec 1 or fus 3 produces embryos that are very highly pigmented, extremely viviparous and with very reduced protein contents. The fus 3 lec 1 double mutant plants produce embryos that morphologically resemble lec 1 but are smaller. These also frequently produce secondary embryos from the suspensor (∼20% of embryos), as do lec 2 fus 3 double mutant seeds, therefore these loci might act synergistically to suppress the embryogenic potential of the suspensor.

**Fig. 2.** Mutations of the ABI 3, FUS 3 and LEC 1 loci disrupt embryo development and activate seedling characteristics prematurely. (a) Phenotypes of mature embryos (seed coats removed). Magnification ×25. (b) Examination of apical regions from the cleared embryos using Nomarski optics reveals premature activation of the apical meristem (AM) and vascular differentiation (V). Positions of cotyledons (C) and axis (A) are indicated. Magnification ×100.
Molecular dissection of embryo maturation

The molecular genetic resources available for both maize and Arabidopsis have allowed the cloning of several of these seed-specific loci. All encoding putative transcription factors, indicating that they function by regulating the transcription of downstream genes. Interestingly, VP 1, ABI 3 and FUS 3 all share several highly conserved domains.

VP 1, ABI 3 and FUS 3 class transcription factor family

The maize VP 1 locus was the first of this family to be cloned, and has been studied in most detail. It encodes a protein with characteristics of a transcription factor, including an amino-terminal acidic domain, and three basic regions (B1–3, Fig. 3). Transposon-induced mutations of VP 1 have proved very useful in characterizing functional domains within the protein. Two alleles (vp 1-c821708 and vp 1-McW) result in non-viviparous quiescent embryos that are anthocyanin deficient and, consequently, separate these two vp 1-derived phenotypes. The vp 1-McW allele contains an insertion that disrupts the B3 domain, demonstrating that this is not required for the repression of premature germination and induction of desiccation tolerance in maize.

Biochemical and molecular studies have shown that the VP 1 protein has two separate functions; both activating and repressing specific promoter activities. It activates transcription from the Zm promoter (EIN is an abundant embryo-maturation-specific protein), and repression of transcription from the promoter of the barley high pl α-amylase gene amy 6-D (Ref. 17). As α-amylase is expressed in the aleurone of germinating seeds, this observation provides an insight into the function of VP 1 in vivo. By performing the dual functions of activation and repression of gene expression in developing seed, the two multiply exclusive processes of embryo maturation and germination are separated.

Biochemical analysis has also allowed detailed delineation of specific functional domains within VP 1. It has been shown that the B2 domain encodes the binding of a broad range of transcription factors to their target DNA elements in vitro, and a low level of non-specific DNA binding has also been demonstrated. Although VP 1 does not contain any recognizable DNA-binding motif, in vitro experiments show that the B3 domain, in isolation, can bind specifically to the 'Sp' element that is involved in VP 1 activation of the C1 gene (which regulates anthocyanin biosynthesis). These observations indicate that in vivo, protein–protein interactions between VP 1 and other transcription-associated proteins might be important in determining VP 1 functionality.

The ABI 3 gene encodes a protein with a high degree of sequence similarity to VP 1 (Ref. 20). This is a significant observation, because vp 1 and abi 3 seeds share similar phenotypes (including insensitivity to ABA, desiccation intolerance and premature abortion of the shoot apical meristem), indicating that the functional requirement for this protein has been highly conserved in flowering plants. More recently, the ABI 3 and VP 1 homologues have been cloned from several species of flowering plants. All are highly conserved, especially in the B2 and B3 domains, suggesting that these regions are important for the function of the protein, and an ancient origin for this gene family.

Severe alleles of abi 3 completely disrupt embryo maturation processes and molecular analysis of the lesions in these alleles has provided information about ABI 3 function. The abi 3-6 allele contains a deletion of the central third of the protein, the abi 3-3 allele contains a premature stop codon located 30 amino-acids before B2 (Ref. 20). Both of these result in extreme phenotypes, including desiccation intolerance and high levels of insensitivity to applied ABA.

The recent molecular cloning of FUS 3 (Ref. 22) has added more weight to the importance of the B2 and B3 domains, because the predicted FUS 3 protein also contains B2- and B3-like domains (Fig. 3). Mutational analysis shows that the intact B3 domain of FUS 3 is required for correct embryo maturation, in contrast to results obtained with VP 1.

LEC 1 and ABI 4 proteins contain functional domains of transcribed genes

The cloning of LEC 1 and ABI 4 genes from Arabidopsis has been reported recently. The ABI 4 locus encodes a protein containing an APETALA 2 putative DNA-binding domain (APETALA 2 is involved in Arabidopsis flower formation), indicating that ABI 4 functions as a transcription factor. The predicted LEC 1 protein is similar to the yeast CAAT box binding factor (CBF) subunit HAP 3, specifically in the DNA-binding and subunit interaction ‘B’ domain. In other kingdoms CBF’s function as heteroligomers, and interact with the CAATbox cis-element often found upstream of many genes. Unlike animal promoters, plant promoters do not typically contain CAAT boxes. It is not known with what transcription factors or with which promoter elements LEC 1 interacts, but it is very unlikely that it has a general role in transcription, particularly because of the seed-specific phenotypes associated with mutations at this locus.

Functional relationships between ABI 3, FUS 3 and LEC 1

Analyses of the expression patterns of ABI 3, LEC 1 and FUS 3 show that they all accumulate during seed development (as predicted in genetic studies), although FUS 3 is also expressed in other tissues (at low levels). Because genetic studies have shown roles for FUS 3 only during seed development, non-seed-specific functions might be masked by overlapping functions of other genes.
that are expressed following embryo development (ABI 4 also shows non-seed-specific expression\(^2\)).

Each of these genes has a different expression pattern during embryo development (Fig. 3), suggesting an overlapping cascade of functions during embryo development that promote the embryo state and simultaneously repress the development of post-germination processes. The expression of LEC 1 is initiated as early as the two-cell stage in embryos and declines following the bent-cotyledon stage\(^14\). Initial expression of FUS 3 and ABI 3 occurs at approximately the globular stage (around two days after flowering\(^22\), FUS 3 expression peaks at mid-embryogenesis and then declines, whereas ABI 3 expression continues to increase until the end of embryo development. The expression of ABI 3 is affected by both the LEC 1 and the FUS 3 loci\(^1\). Analysis of the quantity of ABI 3 protein in seed extracts shows that, compared with wild-type and abi 3 mutant seed, the level of ABI 3 protein is reduced in abi 3 fus 3 and abi 3 lec 1 double mutant seed. Both FUS 3 and LEC 1 might therefore play roles in regulating ABI 3 protein accumulation. As FUS 3 and ABI 3 proteins share structural domains, do they have overlapping functions? Unfortunately, no null allele of fus 3 has been identified, so it is not possible to determine whether there is any genetic redundancy.

As well as activating genes associated with embryo development, genetic evidence shows that all three factors (and VP 1) repress the expression of genes associated with post-germinative processes. Seeds of mutants containing severe alleles of abi 3 show the encroachment of post-embryonic processes during embryo development. It has been demonstrated that the promoter of the chlorophyll a/b binding protein cab 5, which is normally active following germination, is activated in abi 3-3 mutant embryos to a level similar to that observed in wild-type seedlings\(^3\). Transcripts for malate synthase and isocitrate lyase (ICL), glyoxyosomal enzymes normally expressed during germination, have also been shown to accumulate in the vp 1 mutant but not in wild-type embryos, demonstrating a role for VP 1 in repressing the expression of germination-related genes in the developing maize embryo\(^4\). Similarly, embryos of the mutant lec 1-2 have been shown to accumulate mRNA for ICL and a homologue of the carrot EP2 lipid transfer protein, and to prematurely activate promoters for both these proteins during embryo development\(^\text{5}^\text{6}\). However, it has been suggested that these factors do not always act in concert to repress post-germinative gene expression\(^3\). A new member of the MYB gene family (AtMYB13) has been identified, whose expression is de-repressed in both fus 3-1 and lec 1-1, indicating that the gene is normally repressed by the FUS 3 and LEC 1 loci\(^1\). AtMYB13 is expressed in abi 3-4 embryos at a low level, equivalent to the wild type. AtMYB13 therefore represents a novel gene class differently regulated by ABI 3, FUS 3 and LEC 1.

Independent roles for these loci are reinforced by analysis of their functions following ectopic expression in transgenic plants. Ectopic expression of LEC 1 leads to the growth of embryo-like structures from vegetative tissues of transgenic Arabidopsis plants, demonstrating that LEC 1 is sufficient to induce embryonic pathways. It is probably involved in the maintenance of the embryo-state during embryo development, although it is not clear whether it functions directly in the inhibition of germination, or whether this is a secondary consequence of its action to promote the embryo state\(^7\). In contrast to LEC 1, ectopic expression of ABI 3 or FUS 3 in transgenic Arabidopsis does not appear to alter plant development, indicating that neither have autonomous functions in promoting embryo morphology\(^8\) (although application of ABA to plants expressing ABI 3 does result in the transcription of storage protein genes in leaves, this has not been reported for FUS 3).

**Agricultural applications of basic research**

Molecular cloning of the loci discussed here raises the possibility of manipulating their functions for agricultural benefit. For example, somatic embryogenesis is a useful technology for plant breeding. The observation that ectopic expression of LEC 1 leads to embryo formation on vegetative tissue suggests that it might find important applications in this area. The correct transition from embryo development to germination is also an important agronomic trait. Although many loci have been implicated in controlling the initiation of embryo maturation, acquisition of desiccation tolerance and inhibition of premature germination, less is known.
about the molecular and genetic regulation of the maintenance of dormancy in embryos. This is a problem of particular agricultural significance, because a lack of dormancy in cereals can result in the physiological disorder, pre-harvest sprouting (PHS), where, under cool damp growing conditions, germination starts while the seeds are still in the ear. It is possible to use molecular materials and information gained from studies in maize and Arabidopsis to analyse these problems?PHS in cereals is very similar phenotypically to the top mutation in maize (Fig. 4), raising the interesting possibility that PHS in wheat (and other cereals) is caused by the physiological disruption of the wheat VP1 function.

In contrast to PHS, high levels of embryo dormancy are associated with the success of many weed species, for example, wild oat (Avena fatua). Several studies have shown that specific proteins and mRNAs are upregulated in dormant imbibed seeds of weed species (Bromus secalinus) and A. fatua18,19 and wheat20. This demonstrates that specific gene expression patterns are initiated and maintained during dormancy in imbibed mature seeds.

In A. fatua, several inbred lines have been developed that have different, genetically determined dormancy patterns21. These inbred lines have been used in several studies to analyse the molecular mechanisms regulating the maintenance of dormancy. The A. fatua homologue of VP1 (AfVP1) has been cloned and its expression pattern analysed in seeds from different inbred lines in which the levels of dormancy had been manipulated experimentally.22 Expression of AfVP1 is strongly correlated with the level of embryo dormancy (Fig. 5). Freshly harvested seeds from inbred lines with genetically determined high levels of primary dormancy contain high levels of AfVP1 transcripts. Following after-ripening of these seeds, to a level where dormancy of the imbibed seed is significantly reduced, the level of AfVP1 transcript in imbibed seeds is also reduced. If dormancy is re-introduced into these seeds by environmental treatment (secondary dormancy) these seeds, once again, show high levels of expression of AfVP1. These results demonstrate that the level of the AfVP1 transcript in mature A. fatua seed is modulated by the environment acting on the genotype of the seed and closely correlates with the degree of dormancy. These experiments suggest that there is a role for AfVP1 in the maintenance of dormancy in mature imbibed seeds and indicate that VP1 and ABI3 homologues might be useful markers for dormancy in weeds.

What next?

Progress towards identifying the individual genetic elements that regulate embryo maturation and germination has been greatly aided by the cloning of important regulators of these processes. Now that ABI3, VP1, LEC1 and FUS3 have been cloned, it is possible to begin integrated molecular analyses of their functions. The identification of proteins that interact with these transcription factors will be a high priority. For example, double mutant analyses show that the effects of these genes are synergistic, so it will be interesting to analyse whether the proteins can interact with each other physically. Studies with the isolated B3 domain of VP1 show that the effects of these genes are synergistic, so it will be interesting to analyse whether the proteins can interact with each other physically. Studies with the isolated B3 domain of VP1 suggest that it might bind DNA as an oligomer; FUS3 contains a section with a high degree of homology to B3 (Ref. 22), so it is possible that FUS3 and ABI3 directly interact. A recent study showed that a 14-3-3 protein might have a structural role in holding a VP1–EmBP-1 complex together23. Because many developmental processes are controlled by these factors, it is probable that they interact with several different transcription factors (or perhaps other proteins) to exert their effects. In Arabidopsis, it will be important to determine the molecular relationships between these factors, and other loci, such as LEC2, ABI4, ABI3 and ERA1. Further genetic analysis should also reveal other important regulators. Two Arabidopsis mutants, rdo 1 and rdo 2, specifically reduce embryo dormancy, but do not appear to be ABA-related.24 In addition to PHS, other genetic studies in Arabidopsis have identified several potential quantitative trait loci associated with embryo dormancy25 but none of these loci has been cloned.

What genes do these factors regulate in repressing the transition from embryo to seedling? It has been shown that several post-germination-related promoters are activated in these mutants, but it has yet to be demonstrated whether the transcription factors encoded by these loci directly repress these promoters in wild-type plants. Presumably these factors might repress the activity of loci involved in the earliest stages of germination initiation, and so a search for genetic loci that specifically activate germination is important. No such loci have been reported in Arabidopsis or maize, but evidence from the phenotypes of abi 3, lec 1 and fuj 3 mutant seeds suggests that they might represent proteins involved in processes, such as the cell cycle, meristem activation or vascular differentiation.

It is clear that the transition from embryo development to germination is under strict genetic control that also shows a strong interaction with environmental conditions. The challenge remains to determine how these transcription factors function, and how the environment might regulate them. The availability of cloned proteins that regulate this process, backed up by detailed genetic studies of their interactions, paves the way for a more complete molecular understanding of this transition.

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