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The *ABSCISIC ACID–INSENSITIVE3*, *FUSCA3*, and *LEAFY COTYLEDON1* Loci Act in Concert to Control Multiple Aspects of Arabidopsis Seed Development

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Previous studies have shown that recessive mutations at the Arabidopsis *ABSCISIC ACID–INSENSITIVE3* (*ABI3*), *FUSCA3* (*FUS3*), and *LEAFY COTYLEDON1* (*LEC1*) loci lead to various abnormalities during mid-embryogenesis and late embryogenesis. In this study, we investigated whether these loci act in independent regulatory pathways or interact in controlling certain facets of seed development. Several developmental responses were quantified in *abi3*, *fus3*, and *lec1* single mutants as well as in double mutants combining either the weak *abi3-1* or the severe *abi3-4* mutations with either *fus3* or *lec1* mutations. Our data indicate that *ABI3* interacts genetically with both *FUS3* and *LEC1* in controlling each of the elementary processes analyzed, namely, accumulation of chlorophyll and anthocyanins, sensitivity to abscisic acid, and expression of individual members of the 12S storage protein gene family. In addition, both *FUS3* and *LEC1* regulate positively the abundance of the ABI3 protein in the seed. These results suggest that in contrast to previous models, the *ABI3*, *FUS3*, and *LEC1* genes act synergistically to control multiple elementary processes during seed development.

INTRODUCTION

Embryo development in dicotyledonous plants can be divided roughly into several successive phases (Goldberg et al., 1989, 1994; Hughes and Galau, 1989; West and Harada, 1993; Jürgens and Mayer, 1994). The initial phase is one of morphogenesis, during which embryo pattern formation occurs. This is followed by mid-embryogenesis (alternatively called the maturation phase), during which storage macromolecules, including proteins, lipids, and carbohydrates, are synthesized. These nutritive reserves subsequently support the initial growth of the germinating seedling. Finally, late embryogenesis is characterized by the arrest of tissue growth and development, the induction of dormancy, and the acquisition of desiccation tolerance. The mature, desiccated embryo then remains metabolically quiescent until appropriate environmental conditions trigger germination.

The regulatory mechanisms that coordinate the various developmental events of mid-embryogenesis and late embryogenesis remain largely unknown. Substantial evidence supports the involvement of abscisic acid (ABA), but this hormone is unlikely to be the sole regulatory factor involved (Hughes and Galau, 1991; Giraudat et al., 1994; McCarty, 1995; Rock and Quatrano, 1995). However, significant advances have been made in deciphering the genetic control of seed development in the model dicotyledonous plant Arabidopsis. Work in several laboratories has revealed in particular that the *ABA-INSENSITIVE3* (*ABI3*), *FUSCA3* (*FUS3*), and *LEAFY COTYLEDON1* (*LEC1*) loci play prominent roles in controlling mid-embryogenesis and late embryogenesis. Indeed, mutations at any of these individual loci affect multiple processes, including accumulation of storage proteins as well as acquisitions of dormancy and desiccation tolerance (Koornneef et al., 1984; Finkelstein and Somerville, 1990; Meinke, 1992; Ooms et al., 1993; Bäumlein et al., 1994; Keith et al., 1994; Meinke et al., 1994; Parcy et al., 1994; West et al., 1994; Nambara et al., 1995).

Despite these common phenotypes, it has been proposed that, as schematically depicted in Figure 1, *ABI3* acts in regulatory pathways different from those of *FUS3* and *LEC1* (Bäumlein et al., 1994; Keith et al., 1994; Meinke et al., 1994; West et al., 1994). This conclusion was based on two sets of observations. First, additional, distinct phenotypes have been reported for these two classes of mutants. Responsiveness to ABA and chlorophyll breakdown (one of the last events in Arabidopsis embryo development) is markedly inhibited in *abi3* seeds (Koornneef et al., 1984; Nambara et al., 1992, 1995; Ooms et al., 1993) but not in *fus3* and *lec1*

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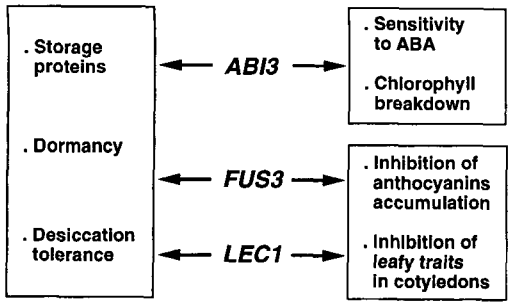

Figure 1. Hypothetical Model with *ABI3*, *FUS3*, and *LEC1* Acting in Independent Regulatory Pathways.

This model accounts for the distinctive phenotypes described in loss-of-function single mutants and for the apparent additivity of these phenotypes in double mutants. Arrows show which wild-type genes are required for the various aspects of normal mid-embryogenesis and late embryogenesis. *FUS3* and *LEC1* are shown here in two different pathways but could belong to a single common pathway equally as well.

(Bäumlein et al., 1994; Keith et al., 1994; Meinke et al., 1994; West et al., 1994). Conversely, unlike the wild type and *abi3* mutants, *fus3* and *lec1* embryos accumulate high levels of anthocyanins and display leafy traits in the cotyledons (Meinke, 1992; Bäumlein et al., 1994; Keith et al., 1994; Meinke et al., 1994; West et al., 1994). Second, the phenotypes of *abi3 fus3* and *abi3 lec1* double mutants appeared to be superpositions of the above-mentioned distinctive phenotypes of the parental single mutants (Bäumlein et al., 1994; Keith et al., 1994; Meinke et al., 1994).

According to the model shown in Figure 1, an *ABI3*-dependent pathway would control positively the sensitivity to ABA and the breakdown of chlorophyll. Distinct *FUS3*- and *LEC1*-dependent pathways would suppress leafy traits in cotyledons and anthocyanin accumulation. In addition, these various pathways would act independently of each other to control storage protein accumulation, seed dormancy, and desiccation tolerance, possibly with each pathway controlling distinct aspects of these complex physiological processes.

However, during preliminary analysis of the *fus3-2 abi3-1* double mutant, it was noticed that the characteristic red color of *fus3* embryos was dramatically enhanced in this double mutant (Bäumlein et al., 1994). This suggested that although accumulation of red anthocyanin pigments had not been observed even in severe *abi3* single mutants, the *fus3* and the weak *abi3-1* mutations had synergistic interactions in enhancing anthocyanin accumulation in double mutant embryos. This intriguing observation prompted us to undertake a more systematic analysis of the possible genetic interactions between *ABI3* and *FUS3* or *LEC1* in controlling seed development.

In this study, we have characterized several double mutants that combine the weak *abi3-1* or the severe *abi3-4* mutations with either a *fus3* or a *lec1* mutation. Various elementary responses (accumulation of chlorophyll and anthocyanin, sensitivity to ABA, and expression of the various members of the 12S storage protein gene family) were quantified in seeds from these double mutants as well as from the parental single mutants. Our phenotypic data indicate that in contrast with the model shown in Figure 1, *ABI3* indeed does interact genetically with both *FUS3* and *LEC1* in controlling each of these elementary developmental processes. In addition, we took advantage of the fact that the *ABI3* gene has been cloned (Giraudat et al., 1992) to investigate whether the above-mentioned genetic interactions might involve modifications in the expression of *ABI3*. Our data indicate that both *FUS3* and *LEC1* positively regulate the abundance of the ABI3 protein in the seed and suggest possible molecular mechanisms that integrate the action of these regulatory loci. Our results are discussed in terms of complementary and synergistic actions of the *ABI3*, *FUS3*, and *LEC1* genes in building a functional network controlling seed development.

RESULTS

Construction of Double Mutants

To investigate the genetic interactions between the *ABI3* and *FUS3* loci as well as between *ABI3* and *LEC1* in controlling seed development, we compared the phenotypes of several single and double mutants. The *abi3-1* (Koornneef et al., 1984) and *abi3-4* (Ooms et al., 1993) single mutants have been induced in the Arabidopsis ecotype Landsberg *erecta* (Ler). The leaky *abi3-1* mutation is a G-to-A transition at position 2143 in the cDNA insert of pcabi3-4F (GenBank accession number X68141) and converts Asp-580 to Asn in the ABI3 protein (C. Valon and J. Giraudat, unpublished results). The severe *abi3-4* mutation converts the codon for Gln-417 into a premature stop codon (Giraudat et al., 1992). The *fus3-2* (hereafter referred to as *fus3*) mutant has been induced in ecotype Dijon (Bäumlein et al., 1994), and the *lec1-1* (hereafter referred to as *lec1*) mutant has been induced in ecotype Wassilewskija (Ws) (Meinke, 1992). The three parental ecotypes, L*er*, Dijon, and Ws, were thus included as controls in our phenotypic studies.

The four distinct double mutants used in this study combined the weak *abi3-1* or the severe *abi3-4* mutation with either the *fus3* or the *lec1* mutations mentioned above. The *fus3 abi3-1* double mutant has been described previously (Bäumlein et al., 1994). The additional *fus3 abi3-4*, *lec1 abi3-1*, and *lec1 abi3-4* double mutants were constructed by crossing homozygous mutant plants, and double mutant embryos were easily identified in immature F_2 seed on the basis of their novel color phenotypes (see below). The genotypes of these double mutants were subsequently confirmed by backcrosses to each of the parental single mutants. Embryos from all of the above double mutants (as well as from *abi3-4*,

fus3, and *lec1* single mutants) are desiccation intolerant, and rescued mutant seedlings were obtained by culturing immature seeds in vitro (see Methods). These double mutant seeds, like the parental single mutants, also failed to become dormant.

Chlorophyll and Anthocyanin Content

The Arabidopsis wild-type embryo is essentially colorless during early development; it then turns green (as a result of chlorophyll accumulation) toward the end of the late-heart stage, remains green until the end of the maturation period, and finally loses color at the onset of desiccation (Jürgens and Mayer, 1994). Figure 2 shows that in the three wild-type ecotypes used in this study (L*er*, Dijon, and Ws), embryos that were excised at the beginning of silique desiccation indeed appeared white. As shown in Figure 3, extracts from the corresponding wild-type seeds contained only trace amounts of chlorophyll (Figure 3A) and no anthocyanin (Figure 3B).

Although *abi3* embryos initially develop in a manner similar to that of the wild type, a characteristic phenotype of severe *abi3* mutants, such as *abi3-4*, is that they remain green in the latest stages of development (Figures 2A and 2B; Ooms et al., 1993; Nambara et al., 1995). Accordingly, at the beginning of seed desiccation, *abi3-4* seeds retained higher levels of chlorophyll than did the wild type (Figure 3A). *abi3-4* seeds also contained a low but spectrophotometrically detectable level of anthocyanins (Figure 3B). The weak *abi3-1* mutant displayed embryo color and chlorophyll content intermediate between those of the wild type and the severe *abi3-4* mutant (Figures 2 and 3A). At this stage, mutant *fus3* embryos did not differ from the wild type in chlorophyll content (Figure

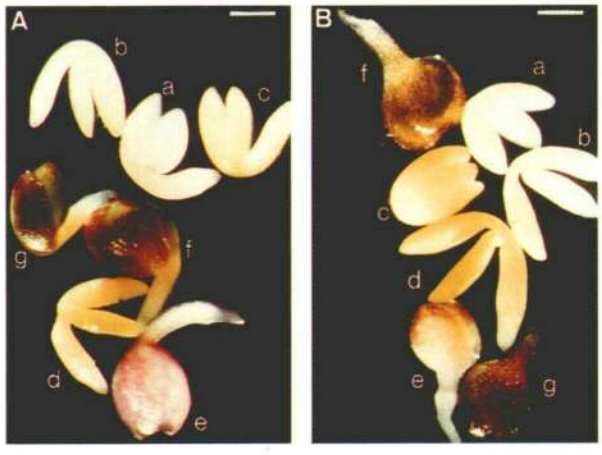

Figure 2. Early Desiccation–Stage Embryos of the Wild Type and Single and Double Mutants.

(A) Embryos dissected from seeds of wild-type L*er* (a) and Dijon (b), from *abi3-1* (c), *abi3-4* (d), and *fus3* (e) single mutants, and from *fus3 abi3-1* (f) and *fus3 abi3-4* (g) double mutants.

(B) Embryos dissected from seeds of wild-type L*er* (a) and Ws (b), from *abi3-1* (c), *abi3-4* (d), and *lec1* (e) single mutants, and from *lec1 abi3-1* (f) and *lec1 abi3-4* (g) double mutants.

Embryos were dissected from immature seeds harvested at the onset of the desiccation phase. Bars = 250 μm.

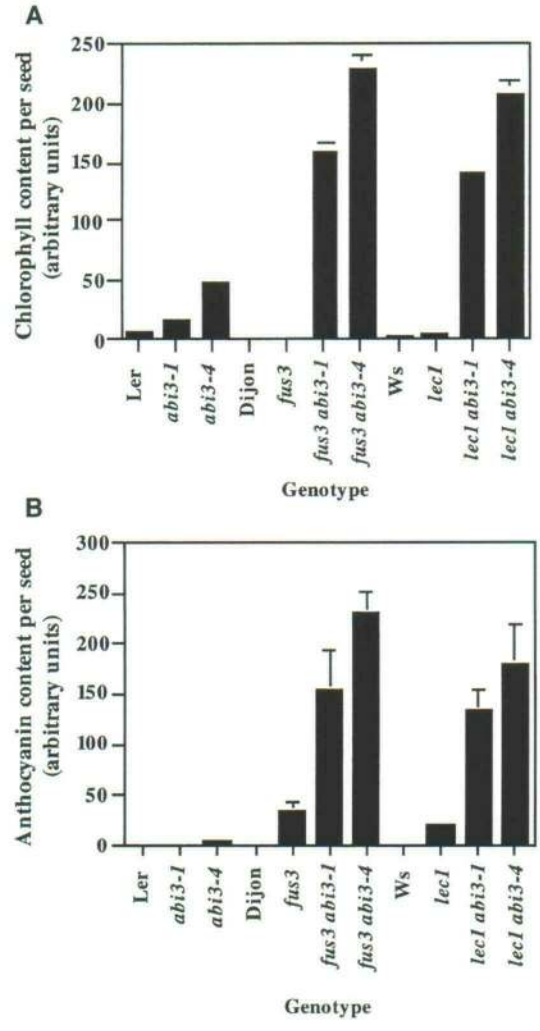

Figure 3. Chlorophyll and Anthocyanin Content of Early Desiccation–Stage Seeds.

Chlorophyll and anthocyanin contents are expressed in arbitrary units and are normalized per seed. For each indicated genotype, values shown are the means of duplicate measurements with samples of 90 to 130 **(A)** or 40 to 70 **(B)** seeds each. Bars represent the range of values obtained.

(A) Chlorophyll content from seeds of wild-type L*er*, Ws, and Dijon, from *abi3-1*, *abi3-4*, *fus3*, and *lec1* single mutants, and from *fus3 abi3-1*, *fus3 abi3-4*, *lec1 abi3-1*, and *lec1 abi3-4* double mutants.

(B) Anthocyanin content from seeds of wild-type L*er*, Ws, and Dijon, from *abi3-1*, *abi3-4*, *fus3*, and *lec1* single mutants, and from *fus3 abi3-1*, *fus3 abi3-4*, *lec1 abi3-1*, and *lec1 abi3-4* double mutants.

3A), but their cotyledons displayed a characteristic pink/red color that was due to the abnormal accumulation of anthocyanin pigments (Figures 2A and 3B; Bäumlein et al., 1994; Keith et al., 1994). *lec1* embryos similarly contained abnormal amounts of anthocyanins, primarily at the tip of cotyledons (Figures 2B and 3B; Meinke, 1992; West et al., 1994). In addition, although this was not obvious in chlorophyll assays with total seed extracts (Figure 3A), the tip of *lec1* cotyledons remained somewhat green unusually late in development (Figure 2B; Meinke, 1992; Meinke et al., 1994).

In all double mutants combining an *abi3* mutation with either the *fus3* (*fus3 abi3-1* and *fus3 abi3-4*) or the *lec1* (*lec1 abi3-1* and *lec1 abi3-4*) mutation, embryos excised at the beginning of seed desiccation displayed a dark color markedly distinct from the phenotypes of the wild type and of the parental single mutants (Figures 2A and 2B). This dark aspect seemed to result from the combination of red and green colors, and spectrophotometric assays confirmed that double mutant seeds indeed contained high levels of both chlorophyll (Figure 3A) and anthocyanin (Figure 3B). However, these quantitative measurements further revealed that in each double mutant, the amounts of chlorophyll (Figure 3A) and anthocyanin (Figure 3B) were at least four times higher than the sum of the amounts in the two parental single mutants. The phenotypes of the double mutants thus indicated synergistic interactions between *abi3* and *fus3* mutations as well as between *abi3* and *lec1* mutations in controlling chlorophyll breakdown and anthocyanin accumulation.

Sensitivity to ABA

Another characteristic phenotype of *abi3* mutants is their reduced sensitivity to the ABA inhibition of seed germination (Koornneef et al., 1984; Ooms et al., 1993; Nambara et al., 1994). To assess the respective impact of *abi3*, *fus3*, and *lec1* mutations on responsiveness to ABA, we analyzed the ability of these single mutants and of derived double mutants to germinate in the presence of various ABA concentrations. Because seeds of most of these mutants are desiccation intolerant, the germination assays were performed with immature seeds excised from siliques at the predesiccation stage. Previous studies with wide ranges of ABA doses revealed that in *abi3* mutants, cotyledon expansion is markedly more resistant to ABA inhibition than is radicle emergence (F. Parcy and J. Giraudat, unpublished observations; also see Figure 4A). Hence, germination was scored here using green and expanded (at least twice the size of the seed coat) cotyledons as a unique criterion for all genotypes. As illustrated in Figure 4, this criterion allowed us to make a clear-cut distinction between germinated seeds (Figures 4A to 4D and 4F) and nongerminated seeds (Figure 4G). This criterion also permitted us to unambiguously differentiate ABA-resistant germination (Figures 4A to 4D and 4F) from seed vivipary (Figure 4E).

As shown in Figure 5, wild-type (L*er*, Dijon, and Ws) seed did not germinate in the presence of ABA. Seeds of the weak *abi3-1* mutant showed partial germination on 10 and 20 μM ABA, and germination of the severe *abi3-4* mutant was totally resistant to all ABA concentrations tested (Figures 5A and 5B). In agreement with previous studies (Bäumlein et al., 1994; Keith et al., 1994; Meinke et al., 1994), *fus3* seed displayed an ABA sensitivity identical to that of the wild type (Figure 5A). In contrast, germination of double mutant *fus3 abi3-1* seed was fully resistant to all ABA doses (Figure 5A). This double mutant thus behaved like the severe *abi3-4* allele and displayed a dramatic reduction in ABA sensitivity compared with both of its *abi3-1* and *fus3* single mutant parents.

Germination of mutant *lec1* seed previously has been reported to be inhibited by micromolar doses of ABA (Meinke et al., 1994; West et al., 1994). Unexpectedly, in these germination assays, *lec1* seeds repeatedly were found to be more resistant to ABA than were wild-type and even *abi3-1* seeds (Figure 5B). In addition, whereas germination of *lec1* seed was partially inhibited by 20 and 50 μM ABA, the *lec1 abi3-1* double mutant was fully resistant to all ABA doses and behaved in this respect like *abi3-4* (Figure 5B). This analysis thus revealed that seeds of the *lec1* single mutant display a reduced sensitivity to ABA and that the *lec1* and *abi3-1* as well as the *fus3* and *abi3-1* mutations have synergistic interactions in decreasing the responsiveness to ABA in seed.

Expression of the 12S Seed Storage Protein Gene Family

In Arabidopsis, the major classes of seed storage proteins are the 12S cruciferins and 2S napins (Heath et al., 1986). Mutant *abi3*, *fus3*, and *lec1* embryos all accumulate reduced amounts of storage proteins and, in particular, of 12S cruciferins (Nambara et al., 1992; Bäumlein et al., 1994; Keith et al., 1994; Meinke et al., 1994). Cruciferins are encoded by a small gene family that consists of three or four members, depending on the Arabidopsis ecotype. The family includes one or two closely related *CRA* genes and the single-copy *CRB* and *CRC* genes (Pang et al., 1988). We investigated whether the *abi3*, *fus3*, and *lec1* mutations reduce the total content in 12S cruciferins by affecting the expression of similar or distinct members of the cruciferin gene family.

The accumulation kinetics of the *CRA*, *CRB*, and *CRC* mRNAs were determined by gel blot analysis of developing siliques in the wild type and single and double mutants. In agreement with a previous report (Pang et al., 1988), the full-length *CRA*, *CRB*, and *CRC* cDNA probes used in this study did not cross-hybridize with each other (data not shown). As illustrated in Figure 6 for *CRB*, expression of all cruciferin mRNAs started at about the middle of seed development (9 days after pollination [DAP] in this batch of plants), then reached a plateau, and finally decreased toward the dry seed stage (18 DAP). For each cruciferin gene, the transcript levels in the various RNA samples from wild-type and mutant

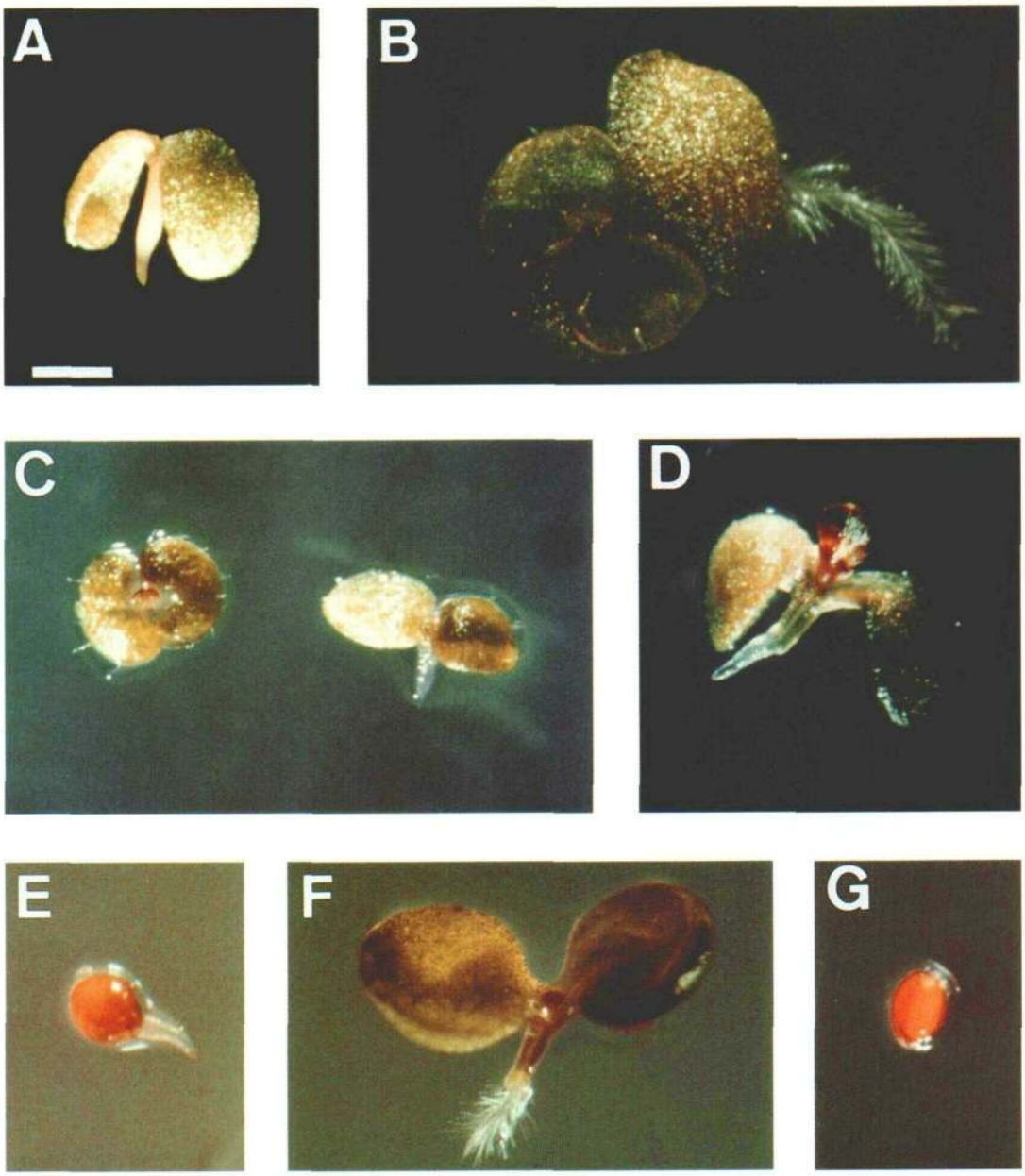

Figure 4. ABA Response of Immature Wild-Type and Single and Double Mutant Seed.

Immature seeds were excised at the predesiccation stage, plated on medium supplemented with 20 μM ABA, and incubated for 12 days at 21°C.

(A) *abi3-1* seedling.

(B) *abi3-4* seedling.

(C) *lec1* seedling.

(D) *lec1 abi3-1* seedling.

(E) *fus3* seed.

(F) *fus3 abi3-1* seedling.

(G) Nongerminated Ws wild-type seed.

The *fus3* seed **(E)** displayed a protruding radicle as a result of vivipary but no cotyledon emergence. All photographs were taken at the same magnification. Bar in **(A)** = 600 μm for **(A)** to **(G)**.

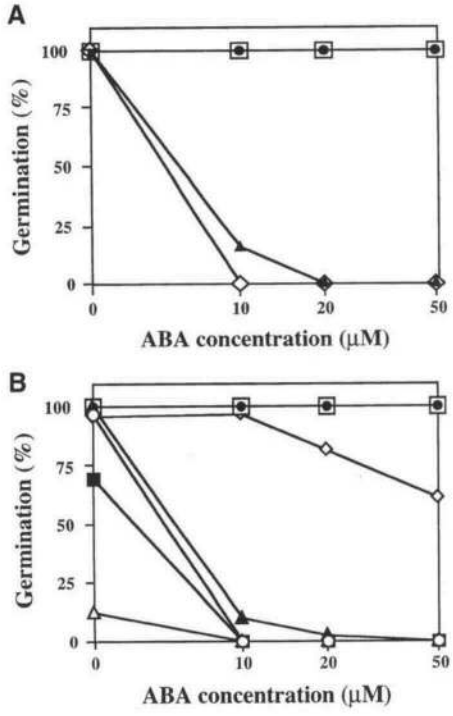

Figure 5. Germination of Wild-Type and Single and Double Mutant Seeds in the Presence of Exogenous ABA.

Immature seeds were excised at the predesiccation stage, plated on medium supplemented with the indicated concentration of ABA, and incubated for 12 days at 21°C. The number of germinated seeds (with expanded green cotyledons) was expressed as the percentage of the total number of seeds plated (25 to 35).

(A) Immature seeds of wild-type L*er* (open diamond) and Dijon (open diamond) and *abi3-1* (filled triangle), *abi3-4* (filled circle), *fus3* (open diamond), and *fus3 abi3-1* (open square) mutants. Because identical values were obtained for L*er*, Dijon, and *fus3* seeds, these three genotypes are all represented here by the same symbol (open diamond) for the sake of clarity.

(B) Immature seeds of wild-type L*er* (filled square) and Ws (open triangle) and *abi3-1* (filled triangle), *abi3-4* (filled circle), *lec1* (open diamond), and *lec1 abi3-1* (open square) mutants. Nonstratified Ws seed (open triangle) germinated poorly on ABA-free medium. Ws seed that were chilled for 3 days at 4°C in darkness to break seed dormancy before incubation at 21°C (open circle) germinated well in the absence but not in the presence of ABA. Also, the rate of germination of L*er* seed on ABA-free medium was lower in **(B)** than in **(A)**, most likely because of differences in the degree of dormancy between these two seed batches.

developing siliques were quantified using a PhosphorImager; the values obtained are shown in Figures 7A to 7C.

Each of the *ABI3*, *FUS3*, and *LEC1* loci appears to participate in controlling the developmental expression of all cruciferin genes. Accumulation of all three cruciferin mRNAs was reduced by at least 50% in each of the *abi3-4*, *fus3*, and *lec1* single mutants (Figure 7). However, the cruciferin genes differed slightly in their respective sensitivity to these various mutations in that each of the three mutations had a more pronounced inhibitory effect on *CRB* than on *CRA* and *CRC*.

The *ABI3* locus appears to interact genetically with both *FUS3* and *LEC1* in controlling *CRB* mRNA accumulation. The sum of the inhibitions of *CRB* expression observed in the *abi3-4* and in the *fus3* single mutants markedly exceeded 100% (Figure 7). Therefore, it seems unlikely that the *ABI3* and *FUS3* loci regulate the abundance of the *CRB* mRNA by acting in two independent (additive) pathways. Similarly, reductions in *CRB* mRNA level in the *abi3-4* and *lec1* single mutants indicate that *ABI3* and *LEC1* do not belong to two independent pathways controlling *CRB* expression (Figure 7).

Our data suggest that developmental regulation of the *CRA* and *CRC* transcripts may also involve interactions between *ABI3* and *FUS3* as well as between *ABI3* and *LEC1*. The *CRA* and *CRC* mRNA levels are significantly higher in *abi3-1* than in *abi3-4* (Figure 7), as a result of the remaining activity of the ABI3-dependent pathway in the weak *abi3-1* mutant. If *ABI3* and *FUS3* were acting independently of each other, this remaining activity of the ABI3-dependent pathway should not have been inhibited by the *fus3* mutation. The *fus3 abi3-1* double mutant should then have contained substantial *CRA* and *CRC* mRNA levels (equivalent to the differences in mRNA levels between *abi3-1* and *abi3-4*), which was not the case (Figure 7). Similar sets of evidence support the idea that *ABI3* and *LEC1* interact in controlling *CRA* and *CRC* expression (Figure 7).

Expression of the ABI3 Protein

Phenotypic characterization of the *fus3 abi3* and *lec1 abi3* double mutants revealed synergistic interactions between

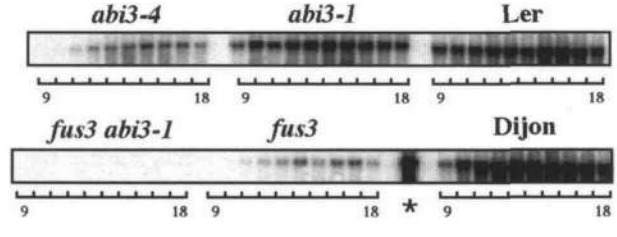

Figure 6. Gel Blot Analysis of *CRB* mRNA Expression during Silique Development.

The *CRB* gene–specific probe was hybridized with total RNA (1 μg) isolated from wild-type L*er* and *abi3-1* and *abi3-4* mutants (top) and from wild-type Dijon and *fus3* and *fus3 abi3-1* mutants (bottom). For each genotype, siliques formed on the same day were pooled, and the samples shown provide a continuous coverage of silique development from 9 DAP to ripeness (18 DAP), as indicated on the scales below the gels. At bottom, the asterisk indicates the RNA sample from 17-DAP L*er* siliques that was included in all blots as an internal standard.

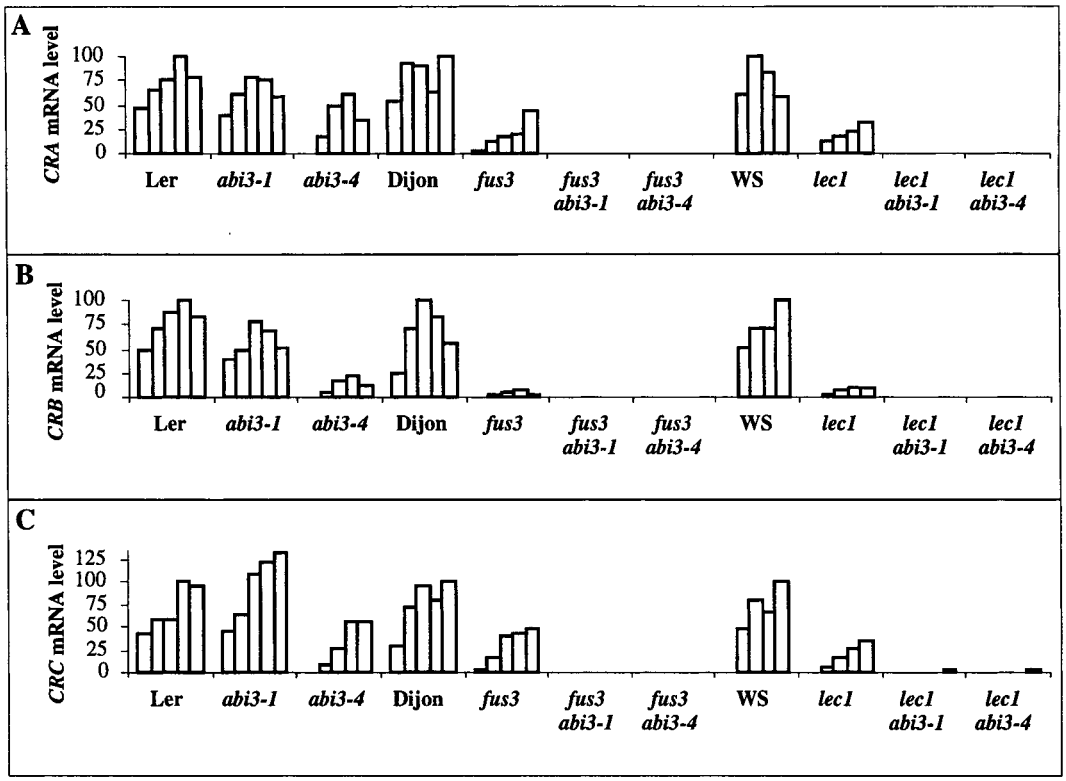

Figure 7. Levels of *CRA*, *CRB*, and *CRC* Cruciferin mRNAs during Silique Development.

Each cruciferin probe was hybridized with RNA blots that provided continuous coverage of the second half of silique development in the various Arabidopsis genotypes (see Figure 6), and hybridization signals were quantified using a PhosphorImager. To limit the size of the graphs, variations in mRNA levels are shown here by 2-DAP intervals rather than by 1-DAP intervals, as shown in Figure 6. For each genotype, the five values shown here thus correspond to the means of the mRNA levels observed in siliques harvested at (from left to right) 9 and 10 DAP, 11 and 12 DAP, 13 and 14 DAP, 15 and 16 DAP, and 17 and 18 DAP, respectively. The mRNA levels are expressed in arbitrary units, with 100 units corresponding to the maximum mRNA level observed during silique development of the appropriate wild-type ecotype: L*er* for *abi3-1* and *abi3-4*; Dijon for *fus3*, *fus3 abi3-1*, and *fus3 abi3-4*; Ws for *lec1*, *lec1 abi3-1*, and *lec1 abi3-4*.

abi3 and *fus3* mutations as well as between *abi3* and *lec1* mutations in regulating the various developmental responses analyzed above. As a first step toward elucidating the molecular mechanisms of these genetic interactions, we investigated whether the *fus3* and *lec1* mutations have any impact on the expression of the ABI3 protein itself.

The ABI3 protein was monitored by gel blot analysis of immature seeds harvested at the predesiccation stage. Because the accumulation of storage proteins (the most abundant proteins in wild-type seed) is affected by each of the *abi3*, *fus3*, and *lec1* mutations, protein extracts from the various genotypes were standardized to each other according to the number of seeds extracted rather than according to the total protein content in the extract. As shown in Figure 8, the wild-type ABI3 and mutant abi3-1 (which differs from ABI3 by a single amino acid substitution) proteins migrated as 116-kD bands, whereas the truncated abi3-4 protein (truncation due to the premature stop codon introduced by this mutation) migrated as an 85-kD band (Parcy et al., 1994). The immune serum detected an additional 56-kD protein band whose abundance in the various genotypes roughly paralleled that of the intact ABI3 protein; thus, this 56-kD polypeptide may possibly be a proteolytic breakdown product of ABI3 (Parcy et al., 1994).

The L*er*, Dijon, and Ws wild-type ecotypes contained similar levels of ABI3 protein (Figures 8A and 8B). The abundance of the ABI3 protein was slightly increased in the *abi3-1* and *fus3* single mutants (Figure 8A). In contrast, the *fus3 abi3-1* double mutant contained markedly less ABI3 protein than did the *fus3* and *abi3-1* parental mutants and the wild-type ecotypes (Figure 8A). Likewise, the ABI3 protein level was clearly reduced in the *lec1 abi3-1* double mutant, whereas the *lec1* single mutant did not seem to differ significantly from the wild type in ABI3 abundance (Figure 8B). The negative

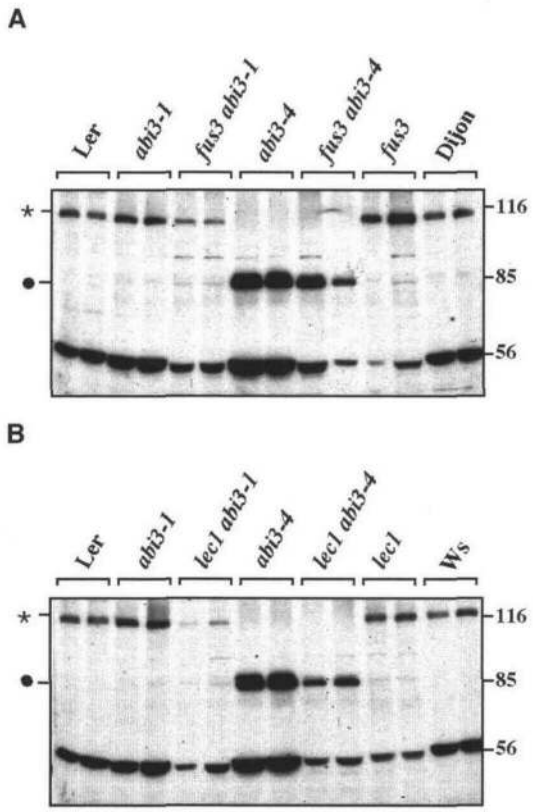

Figure 8. Immunodetection of the ABI3 Protein in Extracts from Predesiccation Seeds.

Each lane of the SDS–polyacrylamide gel was loaded with total protein extracts equivalent to ~40 seeds. Immunodetection was performed using as primary antibodies an immune serum raised against the N-terminal domain of the ABI3 protein. For each genotype, two protein samples derived from independent seed harvests are shown. Apparent molecular masses of the immunoreactive protein bands are shown in kilodaltons at right. At left, the asterisks indicate the positions of the ABI3 and abi3-1 protein bands; the filled dots indicate the positions of the abi3-4 protein.

(A) Seed extracts from wild-type L*er* and Dijon, from *abi3-1*, *abi3-4*, and *fus3* single mutants, and from *fus3 abi3-1* and *fus3 abi3-4* double mutants.

(B) Seed extracts from wild-type L*er* and Ws, from *abi3-1*, *abi3-4*, and *lec1* single mutants, and from *lec1 abi3-1* and *lec1 abi3-4* double mutants.

effect of *fus3* and *lec1* on ABI3 protein level was also seen when these mutations were combined with the *abi3-4* mutation. The *abi3-4* single mutant contained considerably more ABI3 protein than did the wild type. Nevertheless, the level of mutant abi3-4 protein (85-kD band) was much lower in the *fus3 abi3-4* and *lec1 abi3-4* double mutants than in the *abi3-4* single mutant (Figures 8A and 8B).

DISCUSSION

Monogenic *abi3*, *fus3*, and *lec1* mutations lead to common phenotypes, including reductions in storage protein content, seed dormancy, and desiccation tolerance. However, these monogenic mutations also have differential impacts on the sensitivity to ABA, the persistence of chlorophyll in late development, the accumulation of anthocyanins, and the presence of leafy traits in cotyledons. Thus, it was unclear whether the three genes act in independent pathways with partially overlapping functions (Figure 1) or interact in controlling these developmental processes. To distinguish between these two formal possibilities, we quantified several of the above-mentioned phenotypes in single and double mutants. Our data, and in particular, the characterization of double mutants carrying the leaky *abi3-1* mutation, support the idea that *ABI3* interacts genetically with both *FUS3* and *LEC1* in controlling each of the responses analyzed.

ABI3 Interacts with *FUS3* and *LEC1* in Controlling Elementary Developmental Processes

Based on visual examination, the nearly black color of *fus3 abi3-3* (Keith et al., 1994) and *lec1 abi3-3* (Meinke et al., 1994) double mutant embryos has been proposed to be the green color of the chlorophyll of the severe *abi3-3* mutant combined with the red color of anthocyanins of the *fus3* or *lec1* mutant. Our spectrophotometric data of double mutants carrying another severe *abi3* mutation (*abi3-4*) support the idea that the dark color of such double mutant embryos results from the simultaneous presence of high amounts of chlorophyll and anthocyanins. However, this quantitative analysis further demonstrated that the levels of both types of pigments in *fus3 abi3-4* and *lec1 abi3-4* embryos are several times higher than is the simple sum of the levels in the parental single mutants. These synergistic effects were also observed clearly in *fus3 abi3-1* and *lec1 abi3-1* double mutants containing the weak *abi3-1* mutation. Visual examination previously indicated that the amount of anthocyanin in *fus3 abi3-1* embryos is strongly increased compared with *fus3* and *abi3-1* single mutants (Bäumlein et al., 1994). These data confirmed this latter point and further revealed that chlorophyll content is dramatically enhanced in *fus3 abi3-1* embryos as well.

Quantitative assays with the various double mutants analyzed here thus support the idea that *ABI3* genetically interacts with both *FUS3* and *LEC1* in promoting chlorophyll breakdown and in suppressing anthocyanin accumulation during late embryogenesis. The involvement of *ABI3* in inhibiting the accumulation of anthocyanins also is consistent with the presence of low but spectrophotometrically detectable amounts of anthocyanins in the *abi3-4* single mutant (Figure 3B) and with the occasional observation of anthocyanins at the cotyledon margins of *abi3-3* embryos (Nambara

et al., 1995). Similarly, the role of *LEC1* in promoting chlorophyll breakdown is consistent with the persistence of a greenish color in *lec1* cotyledons during the late stage of development (Figure 2B; Meinke, 1992; Meinke et al., 1994).

To compare the ABA sensitivity of desiccation-intolerant and occasionally viviparous mutants, we developed a germination assay with immature seeds, using green and expanded cotyledons as the germination criterion. In this study, the *lec1-1* (*lec1*) single mutation was reproducibly found to entail a substantial resistance to 10 to 50 μM ABA (Figure 5B). This result apparently contradicts previous reports that *lec1-1* and *lec1-2* single mutant seeds had a wild-type sensitivity to micromolar doses of ABA (Meinke et al., 1994; West et al., 1994). Although the exact reasons for these differences are unclear, we noted that West et al. (1994) studied an allele (*lec1-2*) distinct from the one that we used (*lec1-1*) and that Meinke et al. (1994) used root growth rather than cotyledon expansion as a criterion for scoring germinated seeds. However, our data support the idea that, like *ABI3*, the *LEC1* locus positively controls the responsiveness to ABA in seed. Furthermore, the comparison of the germination rates of *abi3-1*, *lec1*, and *lec1 abi3-1* seeds on 20 and 50 μM ABA suggests that the *lec1* and the weak *abi3-1* mutations have synergistic rather than additive effects in reducing the sensitivity to ABA (Figure 5B). Such a synergy could not be observed in the *lec1 abi3-3* double mutant (Meinke et al., 1994), because the severe *abi3-3* mutation itself confers an absolute resistance of up to 300 μM ABA (close to the maximal ABA concentration that can be tested experimentally; Nambara et al., 1994).

Our data from the *fus3-2* (*fus3*) single mutant (Figure 5A) as well as previous studies using different *fus3* mutant alleles and/or lower doses of ABA (Bäumlein et al., 1994; Keith et al., 1994; Meinke et al., 1994) support the idea that *fus3* mutations alone do not alter the inhibition of seed germination by ABA. Nevertheless, characterization of the *fus3 abi3-1* double mutant revealed that the *FUS3* locus does control sensitivity to ABA and interacts genetically with *ABI3* in this process. The extreme ABA resistance of *fus3 abi3-1* seeds can be explained only by synergistic interactions between the *fus3* and the weak *abi3-1* mutations (Figure 5A). Such an enhancement had remained unnoticed in previous double mutant analyses that had been confined to low doses of ABA and/or that involved only the severe *abi3-3* mutant allele (Bäumlein et al., 1994; Keith et al., 1994).

The abundance of all three cruciferin mRNAs was substantially reduced in each of the *abi3-4*, *fus3*, and *lec1* single mutants (Figure 7). These data are qualitatively consistent with previous observations of *CRA* and *CRC* mRNA levels in severe *abi3* mutants (Nambara et al., 1992, 1995; Parcy et al., 1994) and of *CRB* mRNA level in *fus3* mutants (Bäumlein et al., 1994; Nambara et al., 1995). In this study, among the three cruciferin mRNAs, the abundance of *CRB* was most reduced in the severe *abi3-4* mutant (Figure 7). However, *CRB* has been reported previously to accumulate (unlike *CRA* and *CRC*) to a near normal level in the similarly severe *abi3-3* mutant (Nambara et al., 1995). The reason for this discrepancy remains obscure. Nevertheless, our data support the idea that the *ABI3*, *FUS3*, and *LEC1* loci all contribute to the developmental regulation of each of the cruciferin genes. Furthermore, quantitative analysis of individual cruciferin mRNA levels in single and double mutants revealed that *ABI3* interacts with both *FUS3* and *LEC1* in regulating the abundance of the *CRB* mRNA and possibly that of the *CRA* and *CRC* mRNA as well (see Results).

FUS3 and *LEC1* Regulate the Abundance of the ABI3 Protein

Among the three loci analyzed here, *ABI3* is the only one for which the gene has been cloned. Therefore, we investigated whether regulation of the ABI3 protein level might be one of the molecular mechanisms underlying the genetic interactions that were uncovered by the various phenotypic observations discussed above. The abundance of the ABI3 protein was markedly lower in the *fus3 abi3* and *lec1 abi3* double mutants than in the parental *abi3* single mutant (Figure 8). This decrease in (mutant) ABI3 protein content is likely to contribute to the enhancement of the *abi3* phenotypic defects in these double mutants. This result also indicates that at least in the presence of an *abi3* mutation, the *FUS3* and *LEC1* loci regulate positively the level of the ABI3 protein.

However, regulation of the ABI3 protein level appears to be significantly more complex. The abundance of ABI3 was slightly but reproducibly increased in the *fus3* single mutant, which suggests that in a wild-type *ABI3* background, *FUS3* exerts a negative regulation on the ABI3 protein level. Furthermore, ABI3 was also more abundant in *abi3* single mutants, particularly in *abi3-4*, than in the wild type. However, it is unclear whether this latter result reflects the existence of a negative autoregulatory loop affecting ABI3 expression or an increased half-life of the mutant proteins. Preliminary RNA gel blot analysis of the various single and double mutants indicates that the variations in abundance of the ABI3 protein are not systematically correlated with equivalent modifications in the *ABI3* mRNA level. Experiments are in progress to analyze further the mechanisms by which the *ABI3*, *FUS3*, and *LEC1* loci seem to fine-tune *ABI3* expression during seed development.

One may also speculate that the genetic interactions between these three loci involve additional cross-regulation and autoregulation of *FUS3* and *LEC1* gene expression and/or protein stability. Testing this possibility will require the cloning of the *FUS3* and *LEC1* genes.

Toward a Model for the Genetic Regulation of Mid-Embryogenesis and Late Embryogenesis by *ABI3*, *FUS3*, and *LEC1*

As discussed above, the *abi3* and *fus3* as well as the *abi3* and *lec1* mutations had synergistic interactions in regulating each

of the responses analyzed. These results indicate that at least for the developmental processes monitored here, the corresponding loci do not act in independent regulatory pathways. The latter conclusion is reinforced by the finding that both *FUS3* and *LEC1* control the abundance of the ABI3 protein.

For a given phenotype, synergistic enhancement in a double mutant can result from genetic interactions between two loci acting either in a single pathway or in two distinct pathways (Guarente, 1993). To discriminate between these two possibilities, it is essential to know the phenotypes of null mutations in the two interacting genes (Guarente, 1993). Previous phenotypic studies support the idea that *abi3-4* is one of the most severe *abi3* mutant alleles available with respect to ABA sensitivity (Ooms et al., 1993; Nambara et al., 1994) and to the developmental regulation of various marker mRNAs (Parcy et al., 1994; Nambara et al., 1995). Furthermore, the truncated abi3-4 protein lacks two of the four domains that are highly conserved across all known ABI3 orthologs from diverse species (Giraudat et al., 1992; Bobb et al., 1995). However, functional analyses of VIVIPAROUS1, the ortholog of ABI3 in maize, have revealed that the two conserved domains that are deleted in abi3-4 are important for some but not for all of the VIVIPAROUS1 physiological roles (McCarty et al., 1991; Hoecker et al., 1995; McCarty, 1995; Hill et al., 1996). Thus, it seems difficult at this stage to assert that *abi3-4* is also a pseudonull mutation with respect, for instance, to chlorophyll breakdown or anthocyanin accumulation. For most phenotypes analyzed, no major phenotypic variability has been noted within the various *fus3* or *lec1* mutant alleles isolated thus far (Bäumlein et al., 1994; Keith et al., 1994; Meinke et al., 1994; West et al., 1994). However, recent observations indicate that the available *fus3* mutants are unlikely to represent null alleles (S. Miséra, manuscript in preparation). Also, as discussed above, the *lec1-1* allele used here possibly may be more ABA resistant than is the *lec1-2* allele studied by West et al. (1994), whereas, conversely, accumulation of the MADS domain protein AGL15 (for AGAMOUS-like) is markedly inhibited in *lec1-2* embryos but not in *lec1-1* embryos (Perry et al., 1996). In the absence of unambiguous information on the exact severity of the various mutants used here, it seems vain to speculate on whether *ABI3*, *FUS3*, and *LEC1* act in a single or in parallel (redundant) interacting pathways.

Moreover, our phenotypic observations indicate that although each of the various developmental responses analyzed here is jointly regulated by *ABI3*, *FUS3*, and *LEC1*, these responses are not all controlled by the exact same mechanism. All responses were not equally affected by a given mutation. For instance, the *abi3-4* single mutant displayed an extreme reduction in ABA sensitivity but only a slight increase in anthocyanin content. The opposite situation was observed in the *fus3* single mutant. Also, the *lec1* mutant was substantially resistant to ABA but retained only low levels of chlorophyll.

Thus, it appears that as schematically depicted in Figure 9, *ABI3*, *FUS3*, and *LEC1* are key members of an integrated genetic regulatory network of mid-embryogenesis and late embryogenesis. The synergistic interactions observed in the double mutants reinforce that these three genes are involved in controlling each of the elementary responses analyzed and that they have complementary regulatory roles. The single mutant phenotypes further suggest that distinct combinations of these genes may be most effective in controlling different developmental responses. Additional studies are clearly needed to refine this conceptual model and to establish whether or not it can be extended to other facets of seed development. In particular, although we did not detect visually any obvious effect of *abi3* mutations on the presence of leafy traits in the cotyledons of double mutant embryos, this point certainly deserves a more in-depth analysis. It should also be informative to extend, by quantitative measurements, the preliminary characterization of a *fus3 lec1* double mutant reported by West et al. (1994).

The model discussed above is in many respects reminiscent of other experimental systems that involve regulatory networks with cross-talk between regulators and/or regulators

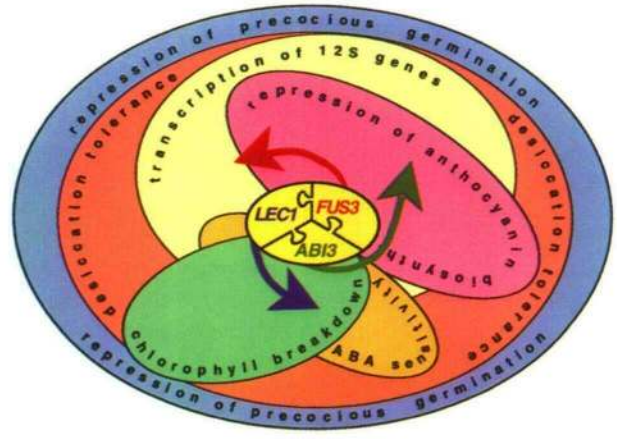

Figure 9. Schematic Model for the Concerted Action of *ABI3*, *FUS3*, and *LEC1* in Controlling Various Aspects of Seed Development.

The three genes are depicted as interlocked jigsaw puzzle pieces to symbolize complementary interactions in regulating seed development, as opposed to models postulating that these genes act in independent regulatory pathways. The jigsaw puzzle represents genetic interactions and does not necessarily imply that the gene products physically interact with each other, although this is an attractive possibility. The various facets of seed development, which are the targets of joint regulation by the three genes, are represented by fields in different colors. Processes that are similarly affected in *abi3*, *fus3*, and *lec1* single mutants are shown in the outer circles. Processes that are differentially affected in the three classes of single mutants are shown as oval fields, and the extent of overlap between an oval field and a given gene increases with the severity of this phenotype in the corresponding single mutant. The arrows originating from the various genes illustrate the synergy between these three loci in controlling each of the developmental responses.

with partially redundant functions (Pickett and Meeks-Wagner, 1995). In animals, members of the MyoD family of myogenic transcriptional regulators display extensive cross-regulations and have both redundant and unique functions (Weintraub, 1993). In Arabidopsis, monogenic mutations in the *APETALA1* (*AP1*), *CAULIFLOWER* (*CAL*), or *LEAFY* genes lead to distinctive phenotypes. Nevertheless, as was revealed by the characterization of double mutants, both *LEAFY* and *CAL* have synergistic interactions with *AP1* in specifying the identity of the flower meristem (Weigel, 1995). Although all of the molecular details of these genetic interactions are not yet known, *AP1* and *CAL* are homologous genes that seem to have partially redundant roles, for instance, in positively controlling the expression of *LEAFY* (Bowman et al., 1993).

Cloning of the *FUS3* and *LEC1* genes should provide some clues to the molecular functions of their protein products, which in turn should help to formulate working hypotheses of the molecular mechanisms underlying the broad model shown in Figure 9. An attractive speculation is that like *ABI3*, *FUS3* and *LEC1* might encode transcription factors. One may then further imagine that these three proteins interact physically and associate into distinct heterooligomeric combinations that regulate different developmental processes. In this hypothetical scenario, the abundance of the ABI3, FUS3, and LEC1 proteins may be controlled by direct actions of these heterooligomers on transcription of the *ABI3*, *FUS3*, and *LEC1* genes as well as by indirect effects of complex formation on the stability of the individual protein components.

Our study sheds new light on the roles of *ABI3*, *FUS3*, and *LEC1* in the control of seed development. Our data indicate that these loci act synergistically rather than independently to regulate multiple, individual developmental processes. They also raise intriguing questions concerning the molecular mechanisms that coordinate the action of these genes and the place of other regulatory loci of seed development, such as *LEC2* (Meinke et al., 1994), in the new genetic model discussed here.

METHODS

Plant Materials and Growth Conditions

The *Arabidopsis thaliana* Landsberg *erecta* (*Ler*) ecotype and the *abi3-1* (isolation number CIV; Koornneef et al., 1984) and *abi3-4* (SM1; Ooms et al., 1993) mutants were provided by M. Koornneef (Wageningen Agricultural University, Wageningen, The Netherlands). The *lec1-1* mutant (Meinke, 1992) and Wassilewskija (Ws) ecotype were obtained from D. Meinke (Oklahoma State University, Stillwater). The *fus3-2* (isolation number 410) single mutant and the *fus3-2 abi3-1* double mutant have been described previously (Bäumlein et al., 1994).

Because most single and double mutant seeds used in this study are desiccation intolerant, homozygous mutant plants were produced by culturing immature embryos on a germination medium containing the inorganic salts of Murashige and Skoog (1962) at half concentrations, 100 mg/L myoinositol, 1 mg/L thiamine–HCl, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine–HCl, 1% sucrose, 0.8% agar, and 2.5 mM 2-(*N*-morpholino)ethanesulfonic acid–KOH, pH 5.7. Rescued mutant seedlings were then transplanted to soil to complete their life cycle.

Plants were routinely grown in a greenhouse (at 22°C with a 16-hr photoperiod) on soil irrigated with mineral nutrients. For comparative phenotypic analyses, we grew all mutants and wild-type controls simultaneously.

Quantification of Chlorophyll and Anthocyanin Pigments

These assays were performed with immature seed harvested at the onset of the desiccation phase. Siliques showing the first signs of desiccation were detached from the mother plant. Seeds were excised from the siliques, counted, and frozen in liquid nitrogen. Frozen seeds were ground at 4°C in 0.2 mL of either 80% aqueous acetone (chlorophyll) or 1% HCl in methanol–H_2O (3:2 [v/v]) (anthocyanins) in a 1.5-mL microcentrifuge tube with a disposable pestle (Lichtenthaler, 1987; Chory et al., 1989). Insoluble material was eliminated by centrifugation at 12,000*g* for 10 min. The absorption spectrum of the supernatant was recorded between either 500 and 700 nm (chlorophyll) or 400 and 650 nm (anthocyanins) in a spectrophotometer. The absorption maximum (found at 663.5 nm for chlorophyll or 533.5 nm for anthocyanins) was subtracted for the baseline, and the quantity of pigment was normalized to the number of seeds used in each sample.

Germination Assays

Germination assays were conducted with immature seeds harvested at the predesiccation stage. On a given inflorescence, the silique showing the first signs of desiccation was identified, and the two (younger) siliques located immediately above that one were harvested and surface sterilized. Seeds were then excised and plated on germination medium agar plates (see above) supplemented with various doses of abscisic acid (ABA). ABA (mixed isomers; Sigma) was diluted from 50 mM stock solutions prepared in methanol; equivalent volumes of methanol were included in the ABA-free controls. Plates were incubated in a growth chamber at 21°C with a 16-hr photoperiod.

RNA Gel Blot Analysis

Siliques were staged by tagging individual flowers on the day of pollination, which was defined as the day the stigma first extruded from the corolla. Intact siliques were harvested on the indicated number of days after pollination (DAP). Total RNA was extracted and subjected to gel blot analysis under stringent conditions as previously described (Parcy et al., 1994). Because several blots were necessary to contain all of the RNA samples derived from the various Arabidopsis genotypes, a sample of RNA from 17-DAP *Ler* siliques was included in all blots as an internal standard. Probes for the cruciferin genes were entire cDNA inserts of clones isolated during systematic analysis of the Arabidopsis transcribed genome (Cooke et al., 1996) and were found to correspond to the *CRA* (GenBank accession number X14312), *CRB* (GenBank accession number X14313), and *CRC* (GenBank accession number U66916) genes, respectively. Filters were then stripped and hybridized with an 18S rRNA probe. Hybridization signals were quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). For each cruciferin probe, the signals were then normalized using the

above-mentioned Ler internal standard to compensate for possible blot-to-blot variabilities in hybridization efficiency and using signals with the 18S rRNA probe to compensate for sample-to-sample heterogeneities in total RNA amount.

Protein Gel Blot Analysis

Seeds were excised from immature siliques harvested at the predesiccation stage (see Germination Assays), counted, and immediately frozen in liquid nitrogen. Total proteins were extracted from 200 to 400 seeds per genotype by using a previously described phenol extraction method (Meyer et al., 1988). Dried pellets were solubilized in 1 × SDS gel-loading buffer (Sambrook et al., 1989), with the volume of the buffer being adjusted to the number of seeds extracted (1 μL per three seeds). Proteins were fractionated by electrophoresis on 8% SDS–polyacrylamide gels and then electroblotted to nitrocellulose membranes (Sambrook et al., 1989). Immunological detection of the ABI3 protein was performed using an enhanced chemiluminescent method (Amersham), using as primary antibodies a polyclonal immune serum raised against the N-terminal domain (Ser-3 to Leu-294) of the ABI3 protein (Parcy et al., 1994).

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