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Regulation of Gene Expression Programs during Arabidopsis Seed Development: Roles of the *ABI3* Locus and of Endogenous Abscisic Acid

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The accumulation kinetics of 18 mRNAs were characterized during Arabidopsis silique development. These marker mRNAs could be grouped in distinct classes according to their coordinate temporal expression in the wild type and provided a basis for further characterization of the corresponding regulatory pathways. The abscisic acid (ABA)-insensitive *abi3-4* mutation modified the expression pattern of several but not all members of each of these wild-type temporal mRNA classes. This indicates that the ABI3 protein directly participates in the regulation of several developmental programs and that multiple regulatory pathways can lead to the simultaneous expression of distinct mRNA markers. The *ABI3* gene is specifically expressed in seed, but ectopic expression of *ABI3* conferred the ability to accumulate several seed-specific mRNA markers in response to ABA in transgenic plantlets. This suggested that expression of these marker mRNAs might be controlled by an *ABI3*-dependent and ABA-dependent pathway(s) in seed. However, characterization of the ABA-biosynthetic *aba* mutant revealed that the accumulation of these mRNAs is not correlated to the ABA content of seed. A possible means of regulating gene expression by developmental variations in ABA sensitivity is apparently not attributable to variations in ABI3 cellular abundance. The total content of ABI3 protein per seed markedly increased at certain developmental stages, but this augmentation appears to result primarily from the simultaneous multiplication of embryonic cells. Our current findings are discussed in relation to their general implications for the mechanisms controlling gene expression programs in seed.

INTRODUCTION

The regulatory mechanisms that ensure the proper execution of seed development remain largely unknown. Several processes essential for seed viability and germination occur during the developmental stages following the morphological pattern formation of the embryo. These processes include the accumulation of protein and lipid reserves, the induction of dormancy, and the acquisition of desiccation tolerance. These developmental stages are characterized by the accumulation of distinct sets of mRNAs (Quatrano, 1988; Goldberg et al., 1989; Hughes and Galau, 1989; Hetherington and Quatrano, 1991; Thomas, 1993; Delseny et al., 1994). Although the exact functions of their protein products are not always firmly established, these mRNAs represent more convenient markers to analyze the regulation of late seed development than the aforementioned complex physiological processes.

In particular, it has been observed that the expression patterns of such marker mRNAs during cotton embryogenesis can be described as combinations of only a few temporal components (Hughes and Galau, 1989). This modularity has been proposed to reflect the existence of sequential gene expression programs, each of them being activated by a distinct global regulatory factor (Hughes and Galau, 1989, 1991). The identity of such factors and corresponding signaling pathways remains to be established. This model of gene regulation during late dicot embryogenesis can be more conveniently tested by genetic means in Arabidopsis than in cotton. A prerequisite to such analyses is the availability of suitable Arabidopsis mRNA markers. In this study, we monitored the expression pattern of multiple marker mRNAs during wild-type Arabidopsis seed development and identified classes of coordinately expressed mRNAs similar to the maturation *MAT* and the late embryogenesis–abundant *LEA* and *LEA-A* temporal classes described in cotton.

These molecular markers were used to investigate further the role of the Arabidopsis abscisic acid (ABA)–insensitive *ABI3* locus in the control of late embryogenesis. Previous studies

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Table 1. Marker mRNAs Monitored during Silique Development: Previously Described Arabidopsis Genes

Gene	Protein Product	Reference
COR47	COR47	Gilmour et al. (1992)
KIN1	KIN1	Kurkela and Franck (1990)
CHS	Chalcone synthase	Feinbaum and Ausubel (1988)
CHI	Chalcone flavanone isomerase	Shirley et al. (1992)
DFR	Dihydroflavonol 4-reductase	Shirley et al. (1992)
At2S3	Napin 3	Guerche et al. (1990)
CRC	Cruciferin C	Pang et al. (1988)
OLEO1	Oleosin 1	van Rooijen et al. (1992)
RAB18	RAB18	Lang and Palva (1992)
AtEm1	AtEm1	Gaubier et al. (1993)
AtEm6	AtEm6	Finkelstein (1993); Gaubier et al. (1993)

demonstrated that seeds of the severe *abi3-3* (Nambara et al., 1992) and *abi3-4* (Ooms et al., 1993) mutant alleles accumulate reduced amounts of storage proteins, remain nondormant, fail to degrade chlorophyll, and do not acquire desiccation tolerance. However, it remained unclear whether the ABI3 protein directly regulates each of these global processes or if the above phenotypic defects indirectly result from the inability of *abi3* mutant embryos to reach the appropriate developmental stage (Nambara et al., 1992; Ooms et al., 1993). We report here that the severe *abi3-4* mutation impairs the accumulation of many but not all representatives of the *MAT*, *LEA*, and *LEA-A* mRNA classes. These mutant seeds thus appear to undergo all developmental stages because they retain the expression of several characteristic mRNA markers.

The possible role of endogenous ABA in the control of seed gene expression is a matter of controversy. The observation that several mRNA markers are precociously inducible by exogenous ABA in cultured embryos from a variety of species prompted the hypothesis that developmental variations in endogenous ABA content play an essential role in regulating gene expression in seed (Quatrano, 1988; Goldberg et al., 1989; Hetherington and Quatrano, 1991; Thomas, 1993). The role of endogenous ABA in the developmental regulation of several ABA-responsive genes was, however, called into question by embryo culture experiments in oilseed rape (Finkelstein and Crouch, 1986), sunflower (Goffner et al., 1990), wheat (Morris et al., 1990), and cotton (Hughes and Galau, 1991). The Arabidopsis *abi3* mutants were originally identified for their ability to germinate in the presence of ABA (Koornneef et al., 1984), and severe *abi3* mutant alleles are several orders of magnitude less sensitive than the wild type to the ABA inhibition of seed germination (Ooms et al., 1993; Nambara et al., 1994). We therefore examined the possible relationship between *ABI3* and endogenous ABA in the control of seed gene expression. We report here that exogenous ABA induces the accumulation of several seed-specific transcripts in transgenic plantlets ectopically expressing *ABI3*. However, characterization of the ABA-deficient *aba-1* mutant revealed that developmental variations in endogenous ABA content are not the major signals triggering the *ABI3*-dependent expression of these same mRNAs during seed development. The respective roles of ABI3 and endogenous ABA are discussed in the context of current models of the regulatory network controlling gene expression in seed.

RESULTS

Kinetic Analysis of Gene Expression during Silique Development in Wild-Type Arabidopsis

The accumulation kinetics of various mRNAs were characterized during silique development in the Landsberg *erecta* wild-type line. A total of 18 Arabidopsis cDNA probes were used. The vast majority of these cDNA clones have been identified during our systematic analysis of Arabidopsis silique (Höfte et al., 1993) or dry seed (M. Raynal and M. Delseny, unpublished data) cDNA libraries. As detailed in Table 1, 11 clones correspond to well-characterized Arabidopsis genes. Most of the remaining clones, listed in Table 2, encode homologs of proteins described in other plant species. Each probe detected a single band upon RNA gel blot analysis.

As shown in Figure 1, the various marker mRNAs monitored here could be grouped in distinct classes according to their coordinate temporal expression in the wild type. Class 1 comprises the cold-regulated *COR47* and *KIN1*, chalcone synthase (*CHS*), chalcone flavanone isomerase (*CHI*), and dihydroflavonol 4-reductase (*DFR*) mRNAs (Figure 1A). These transcripts were detected exclusively during the earliest stages, 0 to 10 days after pollination (DAP), of wild-type silique development. Abundance of the *COR47* and *KIN1* mRNAs was highest during the first days and then progressively decreased, whereas that of *CHS*, *CHI*, and *DFR* mRNAs peaked at ~6 DAP. It is impossible to relate the current class 1 mRNAs to the cotyledon (*COT*) mRNA class defined in cotton (Hughes and

Table 2. Marker mRNAs Monitored during Silique Development: Novel Arabidopsis cDNA Probes

cDNA	Homologous Protein Product	Reference
PAP85	Broad bean vicilin	Bassüner et al. (1987)
PAP38	Rapeseed LEA76	Harada et al. (1989)
PAP51	Cotton LEA D113	Baker et al. (1988)
PAP140	Cotton LEA D34	Baker et al. (1988)
PAP10	Sunflower anther-specific protein	Domon et al. (1990)
M10	Not known	
M17	Not known	

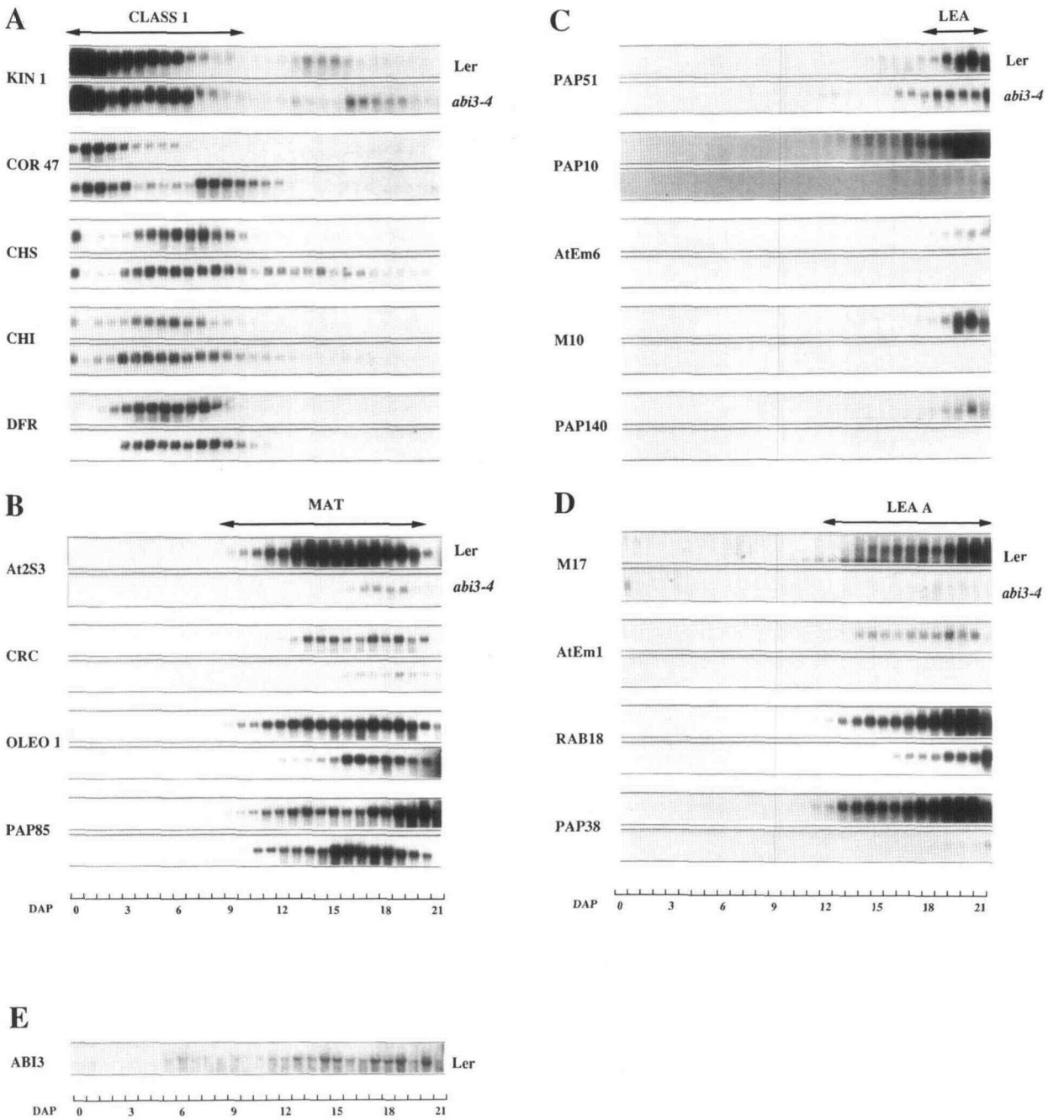

Figure 1. Comparative Gel Blot Analysis of the Expression Pattern of Various mRNAs during Wild-Type and *abi3-4* Silique Development.

Each of the indicated cDNA probes was hybridized to total RNA (**[A]** to **[D]**, 1.5 μg; **[E]**, 5 μg) isolated from Landsberg *erecta* wild-type (Ler, top autoradiogram) and *abi3-4* (bottom autoradiogram) siliques. In this batch of plants grown during winter time, silique development lasted 21 days from anthesis (0 DAP) to dry-seed stage (21 DAP) in both genotypes. The 29 siliques formed during this period were harvested individually and provide a continuous coverage of all stages of silique development.

(A) Class 1 mRNAs.
(B) *MAT* mRNAs.
(C) *LEA* mRNAs.
(D) *LEA-A* mRNAs.
(E) *ABI3* mRNA.

Galau, 1989). It is indeed unclear whether the detected expression of class 1 mRNAs occurs in the embryo, which at these early stages probably contributes only a minor fraction of the total silique RNA probed here.

In wild-type siliques, a second set of transcripts started to accumulate concomitantly with the disappearance of the previously described class 1 mRNAs. Abundance of the Arabidopsis napin *At2S3*, cruciferin *CRC*, and oleosin *OLEO1* mRNAs progressively increased from ~9 DAP, reached a maximum ~15 to 18 DAP, and then decreased until 21 DAP (Figure 1B). The *At2S3* (Krebbers et al., 1988; Guerche et al., 1990) and *CRC* (Pang et al., 1988) mRNAs encode abundant Arabidopsis storage proteins. The *OLEO1* mRNA (van Rooijen et al., 1992) encodes a homolog of the oilseed rape oleosin, which is the prevalent protein component of storage lipid inclusion bodies. These mRNAs are thus typical markers of the *MAT* phase of embryo development (Hughes and Galau, 1989). The *PAP85* cDNA encodes a homolog of the vicilin storage protein from broad bean. This transcript was expressed simultaneously with the above-mentioned *MAT* markers and continued to accumulate during the latest stages of silique development. This two-phase expression resembles that of the *MAT-P* mRNA class (Hughes and Galau, 1989).

The remaining transcripts accumulated later than *MAT* mRNAs and reached their highest levels in the last days of wild-type silique development. In addition, they encode homologs of typical LEA proteins from other species (Tables 1 and 2). These mRNAs could be subdivided into two classes based on their kinetics of expression. The *PAP51*, *PAP10*, *AtEm6*, *M10*, and *PAP140* mRNAs started to accumulate at ~18 DAP, concomitantly with the decline in *MAT* mRNAs (Figure 1C). This set of mRNAs would thus correspond to the *LEA* class (Hughes and Galau, 1989). In contrast, the initial expression of the *M17*, *AtEm1*, ABA-responsive *RAB18*, and *PAP38* mRNAs occurred at ~13 DAP (Figure 1D). This pattern of expression is similar to that of the *LEA-A* class of mRNAs (Hughes and Galau, 1989).

Effects of the *abi3-4* Mutation on Gene Expression during Silique Development

The accumulation kinetics of the same marker mRNAs were analyzed during silique development in *abi3-4* mutant plants grown in parallel with the above wild-type ones (Figure 1). The *abi3-4* mutation significantly modified the expression pattern of at least 13 of the 18 marker mRNAs monitored. Interestingly, this mutation did not equally affect the abundance of all mRNAs belonging to one temporal class as defined in the wild type.

Among class 1 mRNAs (Figure 1A), expression of the *KIN1*, *CHI*, and *DFR* transcripts was essentially identical in both genotypes. The *abi3-4* mutation did not affect the abundance of the *COR47* and *CHS* transcripts during the time period corresponding to their wild-type expression. However, these class 1 mRNAs displayed prolonged expression in *abi3-4* siliques. A second peak of *COR47* expression extended up to 12 DAP, and the *CHS* mRNA was detectable until 20 DAP in *abi3-4*. It remains to be determined whether this *abi3*-specific second phase of *COR47* and *CHS* expression occurs in the same silique tissues as the first phase common to the wild type and *abi3-4*.

Expression of the *At2S3* and *CRC MAT* mRNAs was markedly inhibited in *abi3-4* siliques (Figure 1B). This mutation had a less pronounced effect on the expression of the other *MAT* mRNAs (Figure 1B). Initial accumulation of the *OLEO1* mRNA was delayed in *abi3-4*, but this mRNA then reached maximal levels similar to those in the wild type. Also, the *MAT* temporal component of *PAP85* expression was not significantly altered in *abi3-4*.

The abundance of most representatives of the *LEA* and *LEA-A* mRNA classes was dramatically reduced in *abi3-4* siliques (Figures 1C and 1D). This mutation also markedly inhibited the second (*LEA*-like) phase of *PAP85* expression (Figure 1B). However, accumulation of the *PAP51 LEA* (Figure 1C) and *RAB18 LEA-A* (Figure 1D) mRNAs was only slightly reduced.

This analysis thus revealed that an *ABI3*-dependent pathway(s) contributes, albeit to varying extents, to the correct developmental regulation of numerous marker mRNAs. All the previously mentioned *abi3-4* molecular phenotypes occurred approximately during the last two-thirds of silique development. This is the period during which the Landsberg *erecta* siliques have elevated levels of endogenous ABA (Karssen et al., 1983). This temporal correlation, together with the reduced sensitivity of *abi3-4* germinating seed to exogenous ABA (Ooms et al., 1993), suggested that phenotypes conferred by *abi3-4* studied here might result from a defect in gene regulation by endogenous ABA content. This hypothesis was investigated further using *MAT* (*At2S3* and *CRC*), *LEA* (*AtEm6*), and *LEA-A* (*AtEm1*) representatives that displayed markedly altered expression profiles in *abi3-4* siliques.

Ectopic Expression of *ABI3* Confers the Ability To Accumulate the *At2S3*, *CRC*, and *AtEm1* Seed-Specific Transcripts in Response to ABA Applied to Plantlets

We tested whether the *At2S3*, *CRC*, *AtEm1*, and *AtEm6* mRNAs could indeed be induced by ABA in an *ABI3*-dependent manner, as would be predicted by the aforementioned hypothesis. To eliminate possible interference from endogenous developmental factors, this analysis was performed using an experimental system distinct from developing siliques, namely in vegetative tissues.

The *ABI3* gene is not expressed in vegetative tissues, as was revealed by analyzing transgenic Arabidopsis plants carrying a translational fusion between 5.4 kb of the *ABI3* promoter and the β-glucuronidase (*GUS*) reporter gene. No significant *ABI3*-driven GUS expression could be detected by fluorometric or histochemical assays in the vegetative tissues (roots, leaves, and stems) of adult plants (data not shown). However, as shown in Figure 2B, *ABI3*-driven GUS activity was transiently

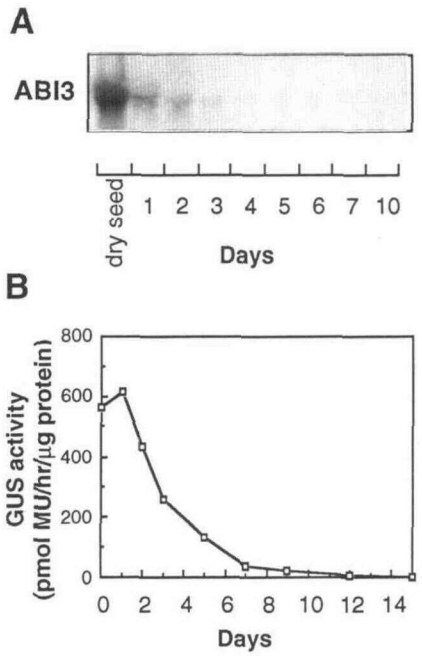

Figure 2. Transient Expression of *ABI3* after Seed Germination.

(A) RNA gel blot analysis. The *ABI3* cDNA probe was hybridized to total RNA (15 μg) isolated from wild-type dry seed and from seedlings harvested at the indicated days after germination.

(B) Fluorometric measurements of *ABI3*-driven GUS activity. GUS activity was measured in *ABI3*::*GUS* and promoterless–*GUS* transgenic seedlings harvested at the indicated days after germination. The promoterless–*GUS* seedlings displayed negligible GUS activity when compared with the *ABI3*::*GUS* seedlings. MU, 4-methylumbelliferone.

present in seedlings during the first week after germination. Histochemical staining revealed that in these young seedlings, the *ABI3*-driven GUS activity was strictly confined to the hypocotyl and cotyledons and was not detectable in the newly formed root or true leaves, as shown in Figures 3H and 3I. The transient presence of *ABI3*-driven GUS activity in young transgenic seedlings did not simply result from the stability of the GUS protein, because the *ABI3* transcript itself was transiently detectable by RNA gel blot analysis of wild-type seedlings (Figure 2A).

Transgenic Arabidopsis plants carrying transcriptional fusions between the double enhanced cauliflower mosaic virus (CaMV) 35S promoter and the *ABI3* cDNA (*EN35S*::*ABI3*) were generated. Two types of *EN35S*::*ABI3* constructs were used (see Methods); similar results were obtained with both. The *EN35S*::*ABI3* transgenic lines did not display any visible alteration in their vegetative development and growth as compared with the wild type. However, a seed phenotype (persistent green color of the embryo and strongly reduced dormancy) very similar to that of severe *abi3* mutant alleles (Nambara et al., 1992; Ooms et al., 1993) segregated in a non-Mendelian fashion in the successive progenies derived from 70% of the primary transformants obtained (data not shown). This phenotype probably reflected cosuppression of the endogenous *ABI3* gene by the transgene (Jorgensen, 1990); however, this point was not investigated further. The transgenic lines consistently segregating for green seed were discarded, whereas some of the lines that recovered a wild-type seed color in future generations were used in the following experiments. Figure 4 shows that such *EN35S*::*ABI3* plantlets displayed a higher sensitivity than did the wild type to the inhibition of root growth by exogenous ABA.

As shown in Figure 5A, *EN35S*::*ABI3* transgenic plantlets expressed the *ABI3* mRNA, whereas in agreement with the *ABI3*::*GUS* data, no *ABI3* transcript was detectable in wild-type plantlets of this age (11 to 13 days). Eleven-day-old transgenic and wild-type plantlets were transferred for two additional days onto plates with or without 50 μM ABA. Even after ABA treatment, the wild-type plantlets contained no detectable amount of the *At2S3*, *CRC*, *AtEm1*, or *AtEm6* mRNAs (Figure 5A). In contrast, the *EN35S*::*ABI3* transgenic plantlets contained a basal level (visible after overexposing the gel blots; data not shown) of *At2S3*, *CRC*, and *AtEm1* mRNAs prior to ABA treatment, and the abundance of these mRNAs markedly increased in response to exogenous ABA (Figure 5A). The *AtEm1* mRNA also accumulated in the rosette leaves of adult greenhouse-grown *EN35S*::*ABI3* plants in response to ABA sprays (Figure 5B). From dilution experiments (data not shown), the *AtEm1* mRNA level in ABA-treated plantlets of transgenic line 19 (Figure 5A) was similar to that found in ripe wild-type siliques. The levels of *At2S3* and *CRC* mRNAs were, however, 1 to 2 orders of magnitude lower in ABA-treated *EN35S*::*ABI3* plantlets than in wild-type siliques during the *MAT* period. No expression of the *AtEm6* mRNA could be detected in any of the *EN35S*::*ABI3* transgenic lines, even after ABA treatment (Figure 5A).

Characterization of the *EN35S*::*ABI3* transgenic lines demonstrated that the *At2S3*, *CRC*, and *AtEm1* mRNAs can indeed be induced by increased ABA levels and that *ABI3* is crucial for this response. These data suggested that the expression of at least these three mRNAs might be subjected to such an *ABI3*-dependent and ABA-dependent regulation in seed. The existence of a possible control mechanism by developmental variations in endogenous ABA levels and/or ABI3 abundance was investigated further.

Expression Pattern of the *At2S3*, *CRC*, *AtEm1*, and *AtEm6* mRNAs during Silique Development in the ABA-Deficient *aba-1* Mutant

Siliques of the *aba-1* biosynthetic mutant (Koornneef et al., 1982; Rock and Zeevaart, 1991) contain less than 5% of the wild-type amount of endogenous ABA (Karssen et al., 1983). We analyzed whether this vast reduction in ABA content has the same effects as the *abi3-4* mutation on the accumulation of the previously mentioned marker mRNAs. Landsberg *erecta* wild-type, *abi3-4*, and *aba-1* mutants were grown simultaneously. Developing siliques were subjected to RNA gel blot analysis; only the relevant stages are shown in Figure 6.

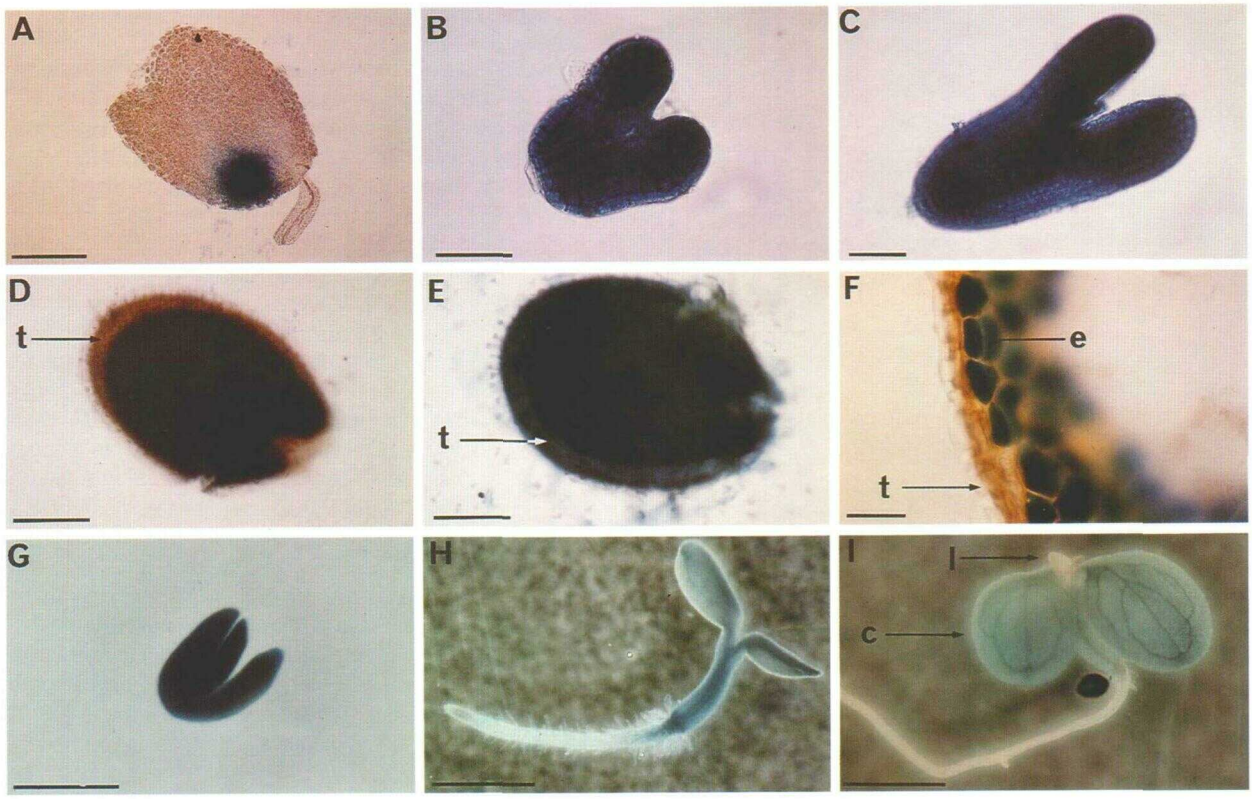

Figure 3. Histochemical Localization of GUS Activity in Transgenic Arabidopsis Plants.

(A) *ABI3*::*GUS* seed containing early-heart stage embryo. Bar = 200 μm.
(B) Isolated mid-heart stage *ABI3*::*GUS* embryo. Bar = 50 μm.
(C) Isolated torpedo stage *ABI3*::*GUS* embryo. Bar = 50 μm.
(D) Testa and endosperm from *ABI3*::*GUS* seed at 15 DAP. Bar = 200 μm.
(E) Testa and endosperm from *EN35S*::*GUS* seed at 15 DAP. Bar = 200 μm.
(F) Section showing the testa and endosperm from an *ABI3*::*GUS* dry seed. Bar = 50 μm.
(G) *ABI3*::*GUS* embryo isolated from dry seed. Bar = 600 μm.
(H) Three-day-old *ABI3*::*GUS* seedling. Bar = 1.2 mm.
(I) Six-day-old *ABI3*::*GUS* seedling. Bar = 2 mm.
t, testa; e, endosperm; c, cotyledon; l, emerging true leaf.

In agreement with data in Figure 1, expression of the *At2S3*, *CRC*, *AtEm1*, and *AtEm6* mRNAs was markedly inhibited in the *abi3-4* mutant. The abundance of the *AtEm1* and *AtEm6* mRNAs was also clearly decreased in *aba-1*, although not as severely as in *abi3-4*. In contrast, the *aba-1* mutation had only minor effects on the expression of the *At2S3* and *CRC* transcripts. The initial accumulation of these mRNAs was possibly slightly reduced, but the *At2S3* and *CRC* mRNAs reached similar maximal levels in *aba-1* and in wild type.

Developmental Regulation of *ABI3* Expression in the Wild Type

We then investigated whether developmental variations in the cellular abundance of the ABI3 protein might trigger a gene expression program(s). The native ABI3 protein was monitored by gel blot analysis. An immune serum was raised against a recombinant protein carrying the N-terminal domain (amino acids 3 to 294) of the ABI3 protein (see Methods). As shown in Figure 7A, this immune serum detected two major protein bands of 116 and 56 kD, respectively, in wild-type seed extracts (lane a). None of these bands were detected by the preimmune serum (data not shown). Two lines of evidence support that the 116-kD protein corresponds to the native ABI3. The *abi3-4* mutation introduces a premature stop codon in the *ABI3* open reading frame (Giraudat et al., 1992). As expected, a lower molecular mass immunoreactive product (85 kD) was found in place of the wild-type 116-kD band in *abi3-4* seed extracts (Figure 7A, lane b). As previously mentioned, RNA gel blot analysis and characterization of *ABI3*::*GUS* transgenic plants demonstrated that *ABI3* is not expressed in wild-type vegetative

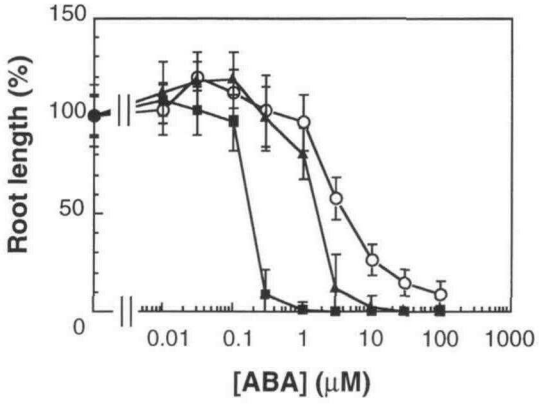

Figure 4. Increased Sensitivity of *EN35S*::*ABI3* Transgenic Plantlets to Root Growth Inhibition by Exogenous ABA.

Seeds of the C24 wild-type (open circles) and *EN35S*::*ABI3* transgenic lines 6 (filled triangles) and 19 (filled squares) were germinated and grown for 5 days on ABA-free medium. These seedlings (with ~5 mm-long roots) were then transferred to plates supplemented with the indicated concentrations of ABA (mixed isomers), and their root lengths were scored after 4 days. The root length of ABA-treated seedlings is expressed as a percentage of those continuously grown on ABA-free medium. Values shown are mean ± SE of samples composed of 12 seedlings each.

tissues. The 116-kD immunoreactive product was absent from wild-type leaf extracts (Figure 7A, lane c) but present in *EN35S*::*ABI3* transgenic leaves (Figure 7A, lane d).

The ABI3 protein was immunologically undetectable in total protein extracts from wild-type seed until 4 DAP (Figure 7B). The amount of ABI3 protein per seed markedly increased between ~5 and 8 DAP and then reached a plateau until the dry-seed stage (16 DAP). The increase in ABI3 level per seed preceded the accumulation of storage proteins, which was detected by Coomassie blue staining starting from 11 DAP (Figure 7C).

The *ABI3*::*GUS* transgenic plants described above permitted a more refined characterization of the *ABI3* expression pattern. Histochemical staining revealed that *ABI3* is expressed in the embryo at all developmental stages examined, that is, from the early-heart to dry-seed stages (Figures 3A to 3C and 3G). The embryo axis and cotyledons appeared evenly stained. Diffuse staining of the endosperm was also observed starting from the torpedo stage. This staining became more obvious in the remaining aleurone layer of the endosperm in mature seed (Figure 3F). In contrast to the *EN35S*::*GUS* positive control (Figure 3E), no significant GUS activity was observed in the seed integuments (Figure 3D) or in the carpels (data not shown) of *ABI3*::*GUS* siliques.

Although detection of the *ABI3* mRNA was limited both by its low abundance and by its comigration with the 25S rRNA, gel blot analysis of wild-type silique total RNA also indicated that the *ABI3* mRNA is expressed throughout silique development (Figure 1E). In *ABI3*::*GUS* transgenic plants, significant *ABI3*-driven GUS activity was detected by fluorometric measurements from 4 DAP until seed ripeness (16 DAP), as shown in Figure 8. *ABI3*-driven GUS activity per seed (and per embryo) increased by 3 orders of magnitude between 4 and 12 DAP. We attempted to evaluate whether this increase in total *ABI3*-driven GUS activity per embryo simply reflected the

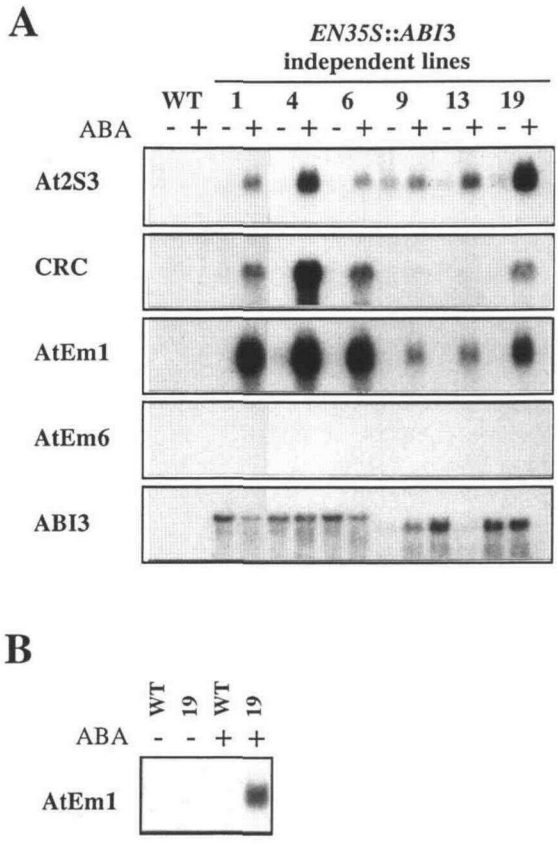

Figure 5. Gel Blot Analysis of mRNAs Induced by ABA in *EN35S*::*ABI3* Transgenic Plants.

(A) The indicated gene-specific probes were hybridized to total RNA (5 μg) isolated from entire plantlets of the C24 wild type (WT) or of independent *EN35S*::*ABI3* transgenic C24 lines (designated by the indicated numbers). Seeds were germinated and grown in vitro for 11 days in the absence of ABA. Plantlets were then transferred for two additional days on plates with (+) or without (−) 50 μM ABA (mixed isomers). As expected, the transcript of the *ABI3* transgene had a smaller size in lines 9 to 19 (transformed with the pD70A construct) than in lines 1 to 6 (transformed with pD70AX) because the *ABI3* untranslated leader had been partially deleted in the construct used to transform lines 9 to 19 (see Methods). For undetermined reasons, the *ABI3* transcript was only barely detectable in untreated plantlets of line 9 and in ABA-treated plantlets of line 13.

(B) The *AtEm1* probe was hybridized to total RNA (5 μg) isolated from the leaves of C24 wild-type or *EN35S*::*ABI3* transgenic (line 19) plants. Greenhouse-grown plants at the rosette stage (before bolting) were sprayed with ABA (+) or with control solution (−) during the days preceding harvest (see Methods).

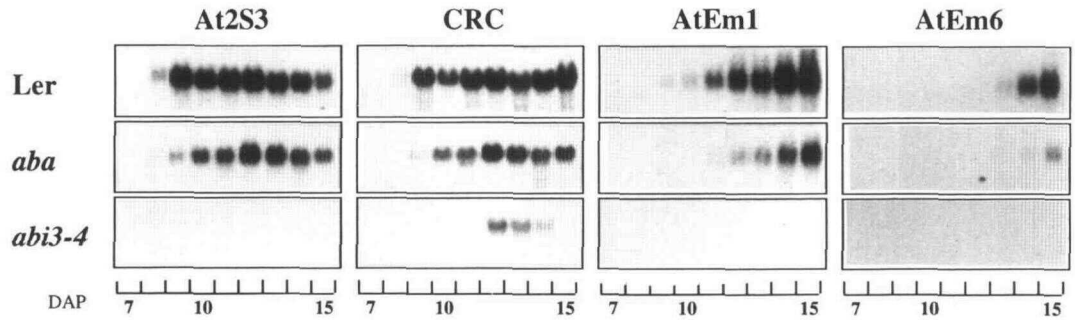

Figure 6. Comparative Gel Blot Analysis of the Expression Pattern of Marker mRNAs during Wild-Type, *aba-1*, and *abi3-4* Silique Development.

Each of the indicated gene-specific probes was hybridized to total RNA (5 μg) isolated from Landsberg *erecta* wild-type (Ler), *aba-1*, and *abi3-4* siliques. In this batch of plants grown during spring time, the dry-seed stage was reached at 15 to 16 DAP in all genotypes. Siliques formed on the same day (approximately two siliques) were pooled, and the nine samples shown provide a continuous coverage of silique development from 7 DAP to ripeness.

simultaneous increase in embryonic cell number. Embryos were thus isolated at distinct morphological stages for which estimates of total cell number have been reported (Jürgens and Mayer, 1994). As shown in Figure 9A, the total *ABI3*-driven GUS activity per embryo increased by a factor of 7 between the early-torpedo (stage 15, ~6 DAP) and bent-cotyledon (stage 18, ~9 DAP) stages. In contrast, the estimated *ABI3*-driven GUS activity per embryonic cell remained essentially constant during this same period (Figure 9B). This suggested that the marked increase in total embryonic *ABI3*-driven GUS activity occurring between 4 and 9 DAP (Figure 8) essentially results from cellular multiplication. The total *ABI3*-driven GUS activity per embryo further increased by a factor of 6 between the bent-cotyledon and mature (stage 19, ~15 DAP) stages (Figure 9A), and a similar increase was observed in the estimated *ABI3*-driven GUS activity per embryonic cell (Figure 9B). However, protein gel blot analysis revealed that the ABI3 level remained essentially constant from 10 DAP until ripeness (Figure 7B). The increase in GUS activity occurring during this particular period might thus reflect the higher stability of the GUS protein as compared with ABI3.

In conclusion, protein gel blot analysis revealed a significant increase in seed ABI3 level only between ~5 and 8 DAP (Figure 7B). The characterization of *ABI3*::*GUS* transgenic plants indicated that this increase in total ABI3 content primarily results from a simultaneous multiplication of embryonic cells. The present data thus suggest that no major variation in ABI3 cellular content occurs during this developmental period that corresponds to the induction of *MAT* mRNAs (*At2S3* and *CRC* in Figure 6).

DISCUSSION

ABI3 Is Specifically Expressed in Seed

The characterization of *ABI3*::*GUS* transgenic plants, together with RNA and protein gel blot analyses of wild-type plants, shows that *ABI3* is expressed throughout seed development. These observations are consistent with the occurrence of *abi3* mutant phenotypes at various stages of seed development (Koornneef et al., 1984; Finkelstein and Somerville, 1990; Nambara et al., 1992; Ooms et al., 1993; this study). The *ABI3* gene is also transiently expressed beyond seed germination in young seedlings but exclusively in the organs of embryonic origin (cotyledons and hypocotyl). In contrast, no *ABI3* expression was detected in the vegetative tissues of seedlings or adult plants grown under standard conditions. Furthermore, we failed to detect any induction of *ABI3* expression in vegetative tissues in response to exogenous ABA, desiccation, or salt treatment (data not shown). Our results provide a straightforward explanation why, unlike *abi1* and *abi2*, *abi3* mutants display no phenotypic alteration in vegetative tissues (Koornneef et al., 1984; Finkelstein and Somerville, 1990; Gilmour and Thomashow, 1991; Nordin et al., 1991; Gosti et al., 1994; Vartanian et al., 1994). Our data do not support the previous conclusion that the *ABI3* gene is active throughout the plant life cycle (Finkelstein and Somerville, 1990). The mutant phenotypes observed in young *abi3-1* seedlings (Finkelstein and Somerville, 1990) might result from the residual *ABI3* expression in cotyledons and/or hypocotyl, or might indicate that the activity of ABI3 during seed development indirectly affects some vegetative responses.

Temporal Programs of Gene Expression during Wild-Type Arabidopsis Silique Development

This study provides a detailed kinetic analysis of gene expression during Arabidopsis silique development. Expression kinetics of the cruciferin and napin gene families had previously been characterized, and our results on the temporal regulation of cruciferin *CRC* and napin *At2S3* mRNAs are in general agreement with these reports (Pang et al., 1988; Guerche et al., 1990). We identified distinct classes of coordinately expressed mRNAs. From both their temporal regulation and the available sequence information on encoded proteins, three of the present classes correspond to the *MAT*, *LEA-A*,

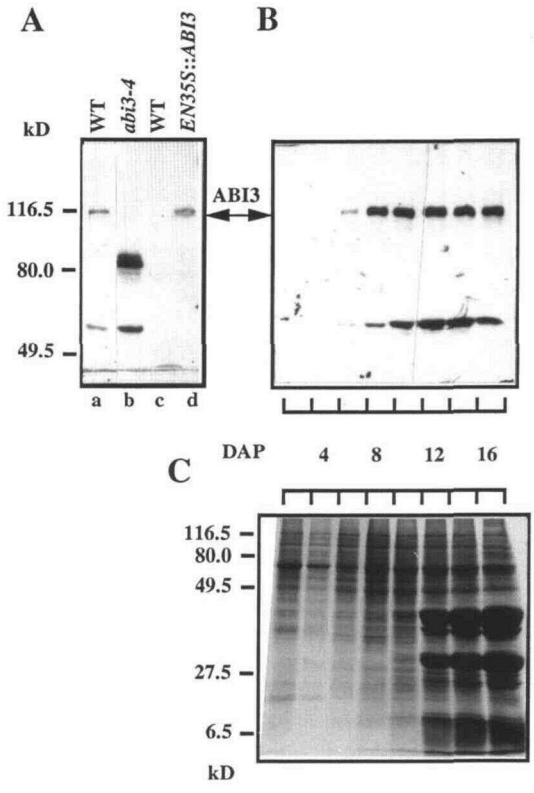

Figure 7. Gel Blot Analysis of the Native ABI3 Protein.

(A) Identification of the 116-kD protein as the native ABI3. The 7% SDS–polyacrylamide gel was loaded with total protein extracts from 14-DAP wild-type (75 μg; lane a) and *abi3-4* (150 μg; lane b) seed, and from wild-type (150 μg; lane c) and *EN35S::ABI3* (150 μg; lane d) rosette leaves. Immunological detection was performed by an enhanced chemiluminescence method using as primary antibodies an immune serum raised against a recombinant ABI3 protein (see Methods). The apparent molecular mass (116 kD) of the native ABI3 on SDS–polyacrylamide gels is higher than the theoretical 79.5 kD predicted from DNA sequence analysis (Giraudat et al., 1992). The highly acidic character of the ABI3 N-terminal region might be responsible for this aberrant electrophoretic mobility. A similar discrepancy between apparent and theoretical molecular masses was indeed observed for a recombinant protein carrying ABI3 residues 3 to 294 but not for a recombinant protein carrying ABI3 residues 362 to 720 (data not shown). WT, wild type.

(B) Expression pattern of the native ABI3 protein during wild-type silique development. Siliques were harvested in eight pools (one per 2 DAP), which provided a continuous coverage from pollination to ripeness (16 DAP). Each lane of the 7% SDS–polyacrylamide gel was loaded with total protein extracts from ~250 seeds isolated from siliques at the various indicated stages. This corresponded to 60 and 400 μg of protein for 8- and 16-DAP-old seed, respectively. Immunological detection was as given in **(A)**.

(C) Coomassie blue staining of total protein extracts from seed. The 12% SDS–polyacrylamide gel was loaded with one fifth (equivalent to ~50 seeds) of the samples used in **(B)**. After electrophoresis, proteins were visualized by Coomassie blue staining. Storage protein bands became predominant in the 12 DAP and subsequent samples. In **(A)** and **(C)**, molecular mass markers are indicated at left in kilodaltons.

and *LEA* mRNA classes defined in cotton embryos (Hughes and Galau, 1989). The *LEA* stage spans only 3 days in Arabidopsis, which precluded further division of this period into distinct postabscission, predesiccation, and desiccation stages as in cotton (Hughes and Galau, 1991).

In contrast, the present class 1 cannot be related with confidence to the *COT* mRNA class defined in cotton embryos (Hughes and Galau, 1989) because, as discussed above, it is unclear whether class 1 mRNAs are expressed in the Arabidopsis embryo. The *KIN1* (Kurkela and Franck, 1990) and *COR47* (Hajela et al., 1990; Gilmour et al., 1992) mRNAs are induced in vegetative tissues in response to dehydration. Whereas many such genes are also expressed during late seed development (Skriver and Mundy, 1990; Delseny et al., 1994), this is clearly not the case for *KIN1* and *COR47*. The expression kinetics of *KIN1* and *COR47* in siliques rather resembles that of the dehydration-inducible *RD22* Arabidopsis mRNA (Yamaguchi-Shinozaki and Shinozaki, 1993).

Representatives of a Single Temporal mRNA Class Are Differentially Affected by the *abi3-4* Mutation

Our data demonstrate that the *abi3-4* mutation inhibits the accumulation of numerous members of the *MAT*, *LEA-A*, and *LEA* temporal mRNA classes. Convergent observations have been reported for a few marker mRNAs in other *abi3* mutant alleles (Pang et al., 1988; Finkelstein and Somerville, 1990; Nambara et al., 1992; Finkelstein, 1993). However, our survey of multiple marker mRNAs further revealed that the *abi3-4* mutation

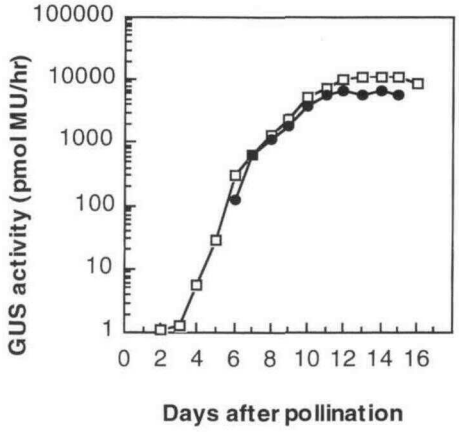

Figure 8. *ABI3*-Driven GUS Activity during Seed and Embryo Development.

GUS activity was assayed in isolated *ABI3::GUS* seeds (open squares) and embryos (filled circles) harvested at the indicated days after pollination. The dry-seed stage was reached at 16 DAP. For each stage, fluorometric measurements were performed on a pool of 10 seeds or embryos. Values shown are normalized per seed or per embryo and have been subtracted for the amount of GUS activity present in corresponding promoterless-*GUS* controls (always less than 2 pmol of 4-methylumbelliferone [MU] per hr per seed or per embryo).

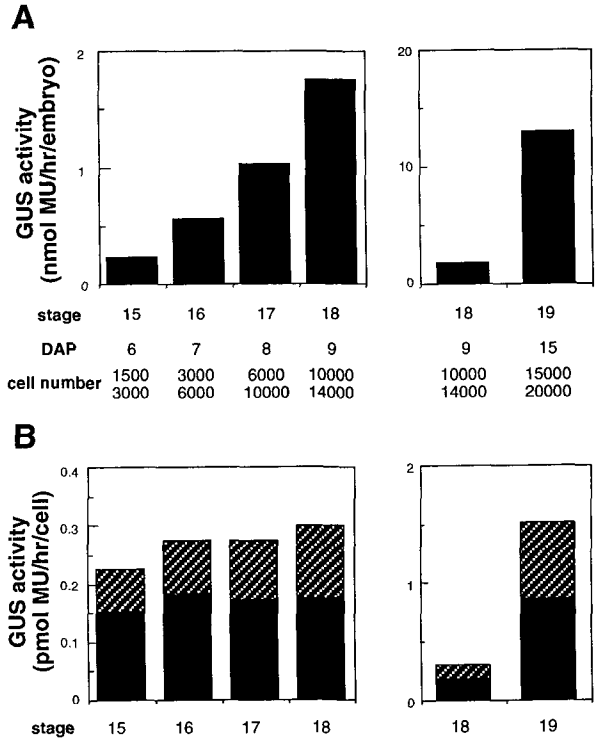

Figure 9. *ABI3*-Driven GUS Activity in Embryos at Distinct Morphological Stages.

(A) GUS activity was measured in isolated *ABI3*::*GUS* embryos. Embryos were examined under a dissecting microscope and staged according to their morphological characteristics. GUS activity was measured on pools of five embryos, and values shown were normalized per embryo. At these stages, promoterless-*GUS* controls contained negligible amounts of GUS activity (see legend to Figure 8). The developmental stages are designated according to the nomenclature of Jürgens and Mayer (1994): early torpedo (stage 15), mid torpedo (16), late torpedo (17), bent cotyledon (18) and mature embryo (19). To facilitate comparison with Figures 6 to 8, approximate days after pollination are indicated for each morphological stage. Also shown are upper and lower estimates of the total embryo cell number at each stage; these values are adapted from Jürgens and Mayer (1994).

(B) Estimates of *ABI3*-driven GUS activity per embryonic cell are shown. These values were calculated from the data given in **(A)**. For each morphological stage, the measured GUS activity per embryo was divided by the upper (black histogram) and lower (hatched histogram) estimates of embryonic cell number.

does not equally affect the accumulation of all mRNAs belonging to a single temporal class. Whereas expression of many mRNAs, such as *MAT At2S3*, *LEA-A AtEm1*, and *LEA AtEm6*, is dramatically impaired in *abi3-4*, some others, such as *MAT PAP85*, *LEA-A RAB18*, and *LEA PAP51*, still accumulate to substantial levels and at roughly the same stages in *abi3-4* as in the wild type.

The severe *abi3-3* (Nambara et al., 1992) and *abi3-4* (Ooms et al., 1993) mutant alleles are similarly impaired in multiple processes characteristic of late seed development, including emergence of seed dormancy, acquisition of desiccation tolerance, and chlorophyll breakdown. Furthermore, the abundance of the few marker mRNAs monitored was found to be drastically decreased in *abi3-3* siliques (Nambara et al., 1992). One of the envisaged interpretations was that seeds of the severe *abi3* mutant alleles display such a variety of phenotypic defects simply because they never reach the corresponding developmental stages (Nambara et al., 1992).

Our findings seem incompatible with that model. In the absence of a true null *abi3* allele, one cannot totally exclude that the mRNAs whose expression is at the most slightly altered in *abi3-4* are nevertheless regulated by the truncated ABI3-4 protein. In this respect, it would be interesting to monitor the expression of these particular marker mRNAs in developing seeds of other available severe *abi3* mutant alleles, such as *abi3-3* (Nambara et al., 1992), *abi3-5* (Ooms et al., 1993), and *abi3-6* (Nambara et al., 1994). However, the unequal effects of the *abi3-4* mutation on accumulation of distinct *MAT*, *LEA-A*, and *LEA* representatives rather suggest that ABI3 is active at each of these stages and that it contributes differentially to the wild-type expression of the multiple associated marker mRNAs. The sustained expression of ABI3 until the dry-seed stage is compatible with this model. Also, our data on *EN35S*::*ABI3* trangenic plants illustrate the ability of ABI3 to affect directly the accumulation of several seed-specific mRNAs. From histochemical localization of *ABI3*-driven GUS activity in seed, the endogenous ABI3 protein probably acts essentially within the embryo (and possibly to some extent in the endosperm) to control the several molecular and physiological aspects summarized earlier. In particular, the absence of detectable *ABI3*-driven GUS activity in the testa does not support the possibility that ABI3 might be indirectly acting from the testa by, for instance, controlling the water status of the seed (discussed by Koornneef et al., 1989).

Respective Roles of *ABI3* and ABA Levels in the Regulation of *At2S3*, *CRC*, *AtEm1*, and *AtEm6* mRNA Expression

The *abi3-4* mutation markedly impairs the accumulation of *At2S3*, *CRC*, *AtEm1*, and *AtEm6* mRNAs during seed development. Mature *abi3-4* seeds are several orders of magnitude less sensitive than the wild type to the inhibition of germination by exogenous ABA (Ooms et al., 1993). Furthermore, developmental variations in seed ABA levels have been traditionally considered as major regulators of gene expression (reviewed in Quatrano, 1988; Goldberg et al., 1989; Hetherington and Quatrano, 1991; Thomas, 1993). The simplest interpretation was thus that accumulation of *At2S3*, *CRC*, *AtEm1*, and *AtEm6* mRNAs in seed is controlled by endogenous ABA content via ABI3.

Characterization of *EN35S*::*ABI3* transgenic Arabidopsis shows that the ectopically expressed ABI3 protein functionally interacts with ABA-signaling cascades in vegetative tissues (inhibition of root growth, gene induction). In particular, ABI3

permits the ABA induction of the *At2S3*, *CRC*, and *AtEm1* mRNAs in *EN35S::ABI3* transgenic plantlets. This result also suggested that the seed-specific expression of ABI3 might in part determine the seed-specific expression of these target mRNAs in wild-type plants.

Wild-type Arabidopsis siliques display two successive peaks of endogenous ABA content. The major peak is mainly of maternal origin and occurs halfway through development; it is followed by an overlapping transient accumulation of zygotic ABA (Karssen et al., 1983). These peaks thus appear to be approximately simultaneous with the onset of the *MAT* and *LEA-A* gene expression programs, respectively. Siliques of the severe *aba-1* mutant allele contain less than 5% of the wild-type amount of endogenous ABA and, in particular, do not display the two successive peaks of ABA content previously described (Karssen et al., 1983).

Nevertheless, the developmental regulation of the *At2S3* and *CRC* mRNA levels was essentially unaltered in *aba-1* siliques. The accumulation of these *MAT* mRNAs thus cannot be controlled by the simultaneous increase in ABA content in the wild type. Alternatively, expression of these mRNAs might conceivably be triggered by an increased ABA sensitivity in embryonic cells. This hypothetical increase in ABA sensitivity is apparently not attributable to developmental variations in ABI3 cellular abundance. The amount of *ABI3*-driven GUS activity and endogenous ABI3 protein per seed did markedly increase simultaneously with the accumulation of *MAT* mRNAs. However, this increase appeared to result primarily from the multiplication of embryonic cells. In any case, a developmental signal(s) distinct from the peak of bulk ABA content must control the expression of *At2S3* and *CRC* mRNAs, and ABI3 is crucial for this response.

Abundance of the *AtEm1* and *AtEm6* mRNAs was significantly reduced in *aba-1* siliques. This reduction is apparently not the result of decreased ABI3 expression, because *aba-1* siliques contain wild-type levels of the *ABI3* transcript (data not shown). The phenotype conferred by *aba-1* indicates that ABA contributes to the wild-type accumulation of the *AtEm1* and *AtEm6* transcripts. Nevertheless, developmental variations in endogenous ABA content do not appear to be the sole factor controlling the expression pattern of these mRNAs. In *aba-1* siliques, the decrease in *AtEm1* and *AtEm6* levels does not seem to be linearly correlated to the vast reduction in ABA content. In the wild type, the *AtEm1* and *AtEm6* mRNA levels increase until seed ripeness, whereas the ABA content decreases before ripeness (Karssen et al., 1983). Finally, the regulatory mechanisms controlling *AtEm1* and *AtEm6* expression must be at least partly different. These mRNAs display distinct temporal expression profiles during seed development, and intriguingly, *AtEm1* but not *AtEm6* mRNA accumulated in ABA-treated *EN35S::ABI3* plantlets. Expression of the *AtEm1* and *AtEm6* mRNAs in wild-type seed thus appears to be regulated by both ABA content and a distinct developmental factor(s). ABI3 most likely participates in these two regulatory components because *AtEm1* and *AtEm6* mRNAs were undetectable in *abi3-4* siliques.

Our data thus indicate that the role of ABI3 is not confined to the ABA pathway(s). ABI3 appears to be required for the action of diverse signals (ABA content and additional developmental factors) controlling the expression of *At2S3*, *CRC*, *AtEm1*, and *AtEm6* mRNAs in developing seed.

Position of ABI3 in the Network Regulating Gene Expression Programs in Seed

The analysis of multiple mRNA markers revealed the existence of similar classes of coordinately expressed mRNAs during cotton and Arabidopsis seed development. These temporal classes thus seem to reflect developmental processes common to dicotyledonous species. Expression of the *MAT*, *LEA*, and *LEA-A* mRNA classes has been proposed to be controlled by a small number of global regulatory factors (Hughes and Galau, 1989, 1991). As previously discussed, our data on the *aba-1* biosynthetic mutant indicate that variations in bulk endogenous ABA content are not the primary signals triggering these programs. A similar conclusion was reached by Hughes and Galau (1991), who, in the absence of appropriate cotton mutants, monitored the expression of multiple marker mRNAs in excised embryos cultured under a variety of conditions. Whereas these authors further proposed that endogenous ABA plays no role in the expression of the *LEA/LEA-A* (or "postabscission") program, the *AtEm1* and *AtEm6* mRNA levels were clearly reduced in *aba-1*. Endogenous ABA content thus probably acts as an additional modulator of the expression of at least some *LEA* and *LEA-A* mRNAs.

Developmental signals (or combinations of signals) distinct from ABA level must therefore be responsible for the specific temporal expression pattern of each of the *MAT*, *LEA-A*, and *LEA* mRNA classes. The *abi3-4* mutation inhibited the accumulation of several representatives from each of these three classes. ABI3 thus seems to participate in multiple regulatory pathways, instead of being confined to the transduction of only one temporal signal in particular. However, the *abi3-4* mutation markedly impaired the accumulation of only some members of each temporal mRNA class. This reveals that at least partially separate signaling pathways control the simultaneous expression of distinct mRNAs. The ABI3 protein appears to contribute to only some of these developmental regulatory cascades.

It is remarkable that despite the differences between dicot and monocot seed development, the Arabidopsis ABI3 and maize VIVIPAROUS-1 (VP1) proteins seem to play similar roles. Like *abi3* mutants, *vp1* mutant seeds display a reduced sensitivity to exogenous ABA (Robichaud and Sussex, 1986), fail to become dormant (Robertson, 1955), and contain severely reduced levels of various storage protein and *LEA/LEA-A* mRNAs (McCarty et al., 1989; Pla et al., 1991; Williams and Tsang, 1991; Paiva and Kriz, 1994). Transient expression studies in protoplasts have demonstrated the ability of the VP1 protein to participate in the ABA induction of gene expression (McCarty et al., 1991; Hattori et al., 1992). However, when the

abundance of marker mRNAs that are totally repressed in *vp1* mutant embryos was compared in various maize ABA-biosynthetic mutant seed, substantial correspondence between transcript level and endogenous ABA content was only rarely observed (Paiva and Kriz, 1994). Thus, similar to ABI3, VP1 probably interacts with various ABA-dependent and ABA-independent pathways regulating gene expression during embryo development. The VP1 and ABI3 proteins display similar architectures and share discrete domains of high amino acid identity (McCarty et al., 1991; Giraudat et al., 1992). Both proteins contain several regions with features previously described in transcriptional activators. In particular, experimental evidence supports that the N-terminal acidic domain of VP1 can indeed participate in transcriptional activation (McCarty et al., 1991). As transcriptional activators, the ABI3 and VP1 proteins could control the intensity of gene expression during seed development by interacting with various transcription factors related to distinct regulatory pathways.

Our data illustrate that ABI3 participates in a complex network regulating the various programs of gene expression during seed development. Future genetic and molecular studies should unravel other elements of this network. Interestingly, several Arabidopsis mutants displaying some phenotypes in common with that conferred by *abi3* have already been described. Available data suggest that the ABI4 and ABI5 proteins may act along the same branch(es) of the network as ABI3 in the control of *AtEm6* mRNA accumulation and of ABA sensitivity (Finkelstein, 1994). The *leafy cotyledon* (*lec1*) (Meinke, 1992) and *fusca3* (*fus3*) (Bäumlein et al., 1994; Keith et al., 1994) mutants are not affected in ABA sensitivity but display other *abi3*-like phenotypes, including reduced accumulation of storage proteins and lipids, lack of dormancy, and desiccation intolerance. The cotyledons of these mutant embryos also show leaf-like morphological characteristics. *lec1* and *fus3* have been described as homeotic and heterochronic mutations, respectively, leading to global defects in the completion of late seed development (Meinke, 1992; Keith et al., 1994). Alternatively, LEC1 and/or FUS3 proteins might (like ABI3) control only a subset of the multiple genes required for global responses such as seed dormancy and desiccation tolerance. As shown here for *abi3*, monitoring of a large set of molecular markers should help to clarify the respective roles of these and other Arabidopsis loci in the control of seed development.

METHODS

Plant Material

The *Arabidopsis thaliana* C24 wild-type line was originally obtained from F. Ausubel (Massachusetts General Hospital, Boston, MA). The Landsberg *erecta* wild-type and derived mutant lines were provided by M. Koornneef (Agricultural University, Wageningen, The Netherlands). The mutant alleles used in this study are *abi3-4* (isolation number SM1; Ooms et al., 1993) and *aba-1* (A26; Koornneef et al., 1982).

Plants were routinely grown in a greenhouse (22°C, 16-hr photoperiod), on soil irrigated with mineral nutrients. Staging of the developing siliques was performed by tagging individual flowers on the day of pollination, which was defined as the day the stigma first extruded from the corolla. Only flowers borne on the primary inflorescence were used. For each stage, total RNA was extracted from a pool of siliques (harvested from ~20 plants grown simultaneously) and was subjected to gel blot analysis.

For aseptic growth, seeds were surface sterilized and plated on germination medium (Valvekens et al., 1988). Petri dishes were incubated for 2 to 4 days in the dark at 4°C, to break seed dormancy, and then in a growth chamber (21°C, 16-hr photoperiod).

Abscisic acid (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. ABA treatment of in vitro grown plantlets was performed by transferring them onto germination medium supplemented with 50 μM ABA. Greenhouse-grown plants at the rosette stage were sprayed with an aqueous 50 μM ABA solution. Sprays were applied three times a day (at 4-hr intervals) during 2 days, and leaves were harvested 30 min after the last treatment.

RNA Gel Blot Analysis

RNA extractions were performed as previously described (Lee Downing et al., 1992). Total RNA was size fractionated on 1% agarose–0.66 M formaldehyde gels (Sambrook et al., 1989) and then transferred to nylon filters (Hybond-N; Amersham) by capillary action with 25 mM $NaPO_4$ buffer, pH 6.5. After UV cross-linking, rRNAs were visualized by methylene blue staining (Sambrook et al., 1989) to verify that equal amounts of RNA were present in each lane. Filters were hybridized at 65°C according to the method of Church and Gilbert (1984), and final washes were performed at 65°C in 20 mM Na_2HPO_4, pH 7.2, 1 mM EDTA, 1% SDS (Church and Gilbert, 1984). Filters were stripped (Church and Gilbert, 1984) for subsequent rehybridization.

^{32}P-labeled DNA probes were generated by random primer extension (Feinberg and Vogelstein, 1983). The Arabidopsis chalcone synthase (*CHS*), chalcone flavanone isomerase (*CHI*), dihydroflavonol 4-reductase (*DFR*), napin *At2S3*, cruciferin *CRC*, ABA-responsive *RAB18*, *AtEm1*, and *AtEm6* mRNAs were detected using the gene-specific probes described in the references listed in Table 1. Entire cDNA inserts were used as probes in the other cases. These latter cDNAs were identified during systematic analysis of the Arabidopsis transcribed genome (Höfte et al., 1993): cold-regulated *COR47* (cDNA clone VBV06-12592; accession number Z18067) and *KIN1* (YAP368; Z17797), oleosin *OLEO1* (YAP230; Z17738), *PAP85* (Z29858 and Z27025), *PAP38* (identical to *PAP002*; Z27249 and Z29837), *PAP51* (Z29850 and Z27256), *PAP140* (Z29875 and Z27037), *PAP10* (identical to *PAP65*; Z27258 and Z29957), *M10* (Z17800), and *M17* (accession number pending).

DNA Constructs

All constructs for plant transformation were made in the pDE1000 binary T-DNA vector (Plant Genetic Systems, Gent, Belgium), which carries a kanamycin resistance marker for plant selection. Recombinant intermediates were cloned into the *Escherichia coli* DH5α strain and verified by restriction digest of plasmid DNA. The final constructs were introduced into the *Agrobacterium tumefaciens* C58C1Rif(pGV2260)

strain by electroporation (Wen-jun and Forde, 1989), and their structures were again verified by restriction digest of plasmid DNA before plant inoculation.

A translational fusion between the abscisic acid–insensitive *ABI3* promoter and the β-glucuronidase (*GUS*) reporter gene was obtained as follows. A 6.4-kb HindIII fragment of the *ABI3* promoter and 5′ leader (ending at the second codon of the *ABI3* open reading frame) was excised from the genomic cosmid 4711 (Giraudat et al., 1992) and cloned in frame with the *GUS* gene in the pBI101.2 vector (Jefferson, 1987). An EcoRI fragment containing 5.4 kb of the *ABI3* 5′ sequence fused to the *GUS* gene and the 3′ untranslated region of the nopaline synthase gene (3′ *NOS*) was then excised and cloned into the EcoRI site of pDE1000 to create the pAG3 plasmid (*ABI3*::*GUS*). The *ABI3*/*GUS* junction was verified by nucleotide sequence analysis. The negative control pGU plasmid (promotorless–*GUS* construct) was obtained by excising the *GUS*::3′ *NOS* cassette from pBI101.2 as a HindIII-EcoRI fragment and cloning it into pDE1000. The pCA2NOS vector (provided by P. Berthomieu, Institut National de la Recherche Agronomique, Versailles, France) contains an overexpression cassette (*EN35S*::3′ *NOS*) composed of the double-enhanced cauliflower mosaic virus (CaMV) 35S promoter (*EN35S*) (Kay et al., 1987) and the 3′ *NOS*. *EN35S* was excised from pCA2NOS as a HindIII-SalI fragment and cloned upstream of the *GUS* gene into pGU, generating the positive control p7G plasmid (*EN35S*::*GUS*).

Constructs for constitutive expression of the *ABI3* transcript were obtained as follows. The *EN35S*::3′ *NOS* cassette was excised from pCA2NOS as a HindIII-BglII fragment and cloned between the HindIII and BamHI sites of pDE1000, generating the pDE1070 plasmid. The almost entire *ABI3* cDNA insert was excised from the pcabi3-4F plasmid (Giraudat et al., 1992) as an XbaI (nucleotide 36 of the cDNA insert)-SalI (located 3′ to the poly[A] tail in the polylinker of the pBluescript SK– vector) fragment. This fragment was inserted between the pDE1070 XbaI and SalI sites located at the junction between the *EN35S* and 3′ *NOS* regions, generating the pD70AX plasmid. The untranslated leader sequence of the *ABI3* cDNA contains several spurious ATG codons (Giraudat et al., 1992). The corresponding region was eliminated from pD70AX by excising an XbaI-HpaI fragment (corresponding to nucleotides 36 to 284 of the pcabi3-4F cDNA insert) and recircularizing the plasmid by blunt end ligation, thereby generating the pD70A plasmid.

Transgenic Plants

Root explants of the Arabidopsis C24 line were transformed with Agrobacterium and regenerated as previously described (Valvekens et al., 1988), except for the following few modifications. Phytagel (Sigma) was used instead of Bacto agar during the cocultivation and regeneration steps. After cocultivation, Agrobacteria were counterselected using 400 mg/L Augmentin (Beecham Laboratories, Nanterre, France). Rooting of the regenerating shoots was induced by a 1-day incubation on germination medium (GM) supplemented with 2 μg/mL α-naphthaleneacetic acid (Sigma). Rooted primary transformants (T_1) were transferred to soil and grown in the greenhouse. For each T_1 line, T_2 seeds were plated on 50 mg/L kanamycin to assay the segregation ratio of kanamycin resistance. The ploidy level of T_2 plants was measured by flow cytometry (Brown et al., 1991). Diploid transgenic lines carrying T-DNA insertions at a single genetic locus were selected, and T_2 progenies homozygous for kanamycin resistance were used for subsequent studies.

Fluorometric Measurement and Histochemical Localization of GUS Activity

Soluble extracts of plant tissues were assayed for GUS activity by fluorometric measurements using 4-methylumbelliferyl glucuronide (Sigma) as a substrate (Jefferson, 1987). Protein concentrations in extracts were determined by the Bradford method (Bradford, 1976). Histochemical localizations were performed using 5-bromo-4-chloro-3-indoyl glucuronide (X-gluc, Biosynth AG, Switzerland) as substrate (Jefferson, 1987). Reactions were conducted at 37°C in the dark for 2 to 8 hr. For all GUS assays on *ABI3*::*GUS* transgenic plants, control experiments were performed in parallel on promoterless–*GUS* and *EN35S*::*GUS* transgenic plants.

Production of Immune Serum against the ABI3 Protein

A recombinant protein containing the N-terminal domain of ABI3 was produced in *E. coli*. The HindIII fragment corresponding to nucleotides 411 to 1487 of the pcabi3-4F cDNA insert (Giraudat et al., 1992) was cloned into the HindIII site of the pBluescript SK– vector (Stratagene). The DNA fragment extending from the pBluescript SK– EcoRV site to the DraI site located at nucleotide 1286 in the *ABI3* cDNA was excised and inserted by blunt end ligation into the SmaI site of the pGEX-2T expression vector (Smith and Johnson, 1988). This recombinant plasmid was introduced into the *E. coli* DH5α strain, and the glutathione *S*-transferase/ABI3 junction was verified by nucleotide sequence analysis. Production of the recombinant protein (carrying ABI3 amino acids Ser-3 to Leu-294) was induced by isopropyl β-D-thiogalactopyranoside (Smith and Johnson, 1988). *E. coli* cells were lysed as previously described (Smith and Johnson, 1988), except that 5 mM DTT and 0.2 mM 4-(2-aminoethyl)benzene-sulfonyl fluoride (AEBSF; Sigma) were included in the lysis buffer. The recombinant protein was affinity purified (Smith and Johnson, 1988) on glutathione Sepharose 4B resin (Pharmacia) and eluted by adding to the resin an equal volume of 2 × SDS gel-loading buffer (Sambrook et al., 1989) supplemented with 0.2 mM AEBSF. The recombinant protein was further purified by preparative SDS-PAGE on 8% polyacrylamide gels (Sambrook et al., 1989) and used for subcutaneous injections of rabbits (Coligan et al., 1993). Preimmune and immune sera were obtained as previously described (Coligan et al., 1993).

Protein Gel Blot Analysis

Total proteins were extracted from plant material by a previously described phenol extraction method (Meyer et al., 1988). Dried pellets were solubilized in 2 × SDS gel-loading buffer (Sambrook et al., 1989) supplemented with 0.2 mM AEBSF. Protein concentrations were determined by the Bradford method (Bradford, 1976). Proteins were fractionated by SDS-PAGE and then electroblotted to nitrocellulose (Schleicher & Schüll) membranes (Sambrook et al., 1989). Molecular weight standards (Bio-Rad) were run in a parallel lane of the SDS–polyacrylamide gel and stained with Coomassie Brilliant Blue R 250. Immunological detection of the ABI3 protein was performed by an enhanced chemiluminescence method (Amersham). The immune serum was preabsorbed with nitrocellulose-immobilized extracts of *E. coli* expressing the glutathione *S*-transferase protein to reduce cross-reactivity. Anti-rabbit horseradish peroxidase–labeled secondary antibodies were used according to the supplier's instructions (Amersham), except that low fat milk (5%) was added as blocking agent.

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