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PEI1, an Embryo-Specific Zinc Finger Protein Gene Required for Heart-Stage Embryo Formation in Arabidopsis

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We used virtual subtraction, a new gene isolation strategy, to isolate several genes of interest that are expressed in Arabidopsis embryos. These genes have demonstrated biological properties or have the potential to be involved in important biological processes. One gene isolated by virtual subtraction is *PEI*. It encodes a protein containing a Cys_3His zinc finger domain associated with a number of animal and fungal transcription factors. In situ hybridization results showed that *PEI1* is expressed throughout the embryo from globular to late cotyledon stage. Transgenic Arabidopsis plants expressing a *PEI1* antisense gene produced white seeds in which embryo development did not progress through heart stage. Aberrant embryos failed to form cotyledons, but the embryonic root appeared to be normal. Aberrant embryos did not turn green, and the expression of genes involved in photomorphogenesis was drastically attenuated. In culture, aberrant embryos did not form true leaves, but root formation was apparently normal. These results suggest that PEI1 is an embryo-specific transcription factor that plays an important role during Arabidopsis embryogenesis, functioning primarily in the apical domain of the embryo.

INTRODUCTION

The architecture of the mature plant is established during embryogenesis. In Arabidopsis, the first asymmetric division of the zygote yields a large basal cell and a small apical cell. The apical cell develops into the embryo proper, which consists of two cotyledons, the shoot apex, the hypocotyl, and the embryonic radicle. The basal cell gives rise to the suspensor, which degrades during later embryo development, and part of the root apex (Mansfield and Briary, 1991). The embryo also has the capability to maintain dormancy under unfavorable conditions and to germinate once suitable conditions occur. Many studies have focused on the molecular mechanism of plant embryo development (Goldberg et al., 1989, 1994; de Jong et al., 1993; Thomas, 1993; West and Harada, 1993; Laux and Jürgens, 1997).

Genetic approaches have been employed to study embryogenesis in Arabidopsis, maize, and rice (Sheridan and Clark, 1993; Hong et al., 1995). Numerous Arabidopsis embryo-defective mutants have been identified using various mutagenesis and screening approaches. Many mutant embryos identified by screening siliques are arrested at late globular stage, implying that the globular- to heart-stage transition is a critical and complex differentiation process in which many genes are required (Feldmann, 1991; Meinke, 1991; Feldmann et al., 1994). Different classes of mutants influencing plant pattern formation have also been identified by screening M_1 seedlings (Jürgens et al., 1991; Mayer et al., 1991). Several embryo mutants have been studied morphologically, for example, *fusca* (*fus*), *leafy cotyledon* (*lec*), *gnom*, *knolle*, and *raspberry* (Mayer et al., 1991, 1993; Meinke, 1991; Castle and Meinke, 1994; Meinke et al., 1994; West et al., 1994; Yadegari et al., 1994); however, only a few genes corresponding to these mutants have been cloned and characterized.

The gene responsible for the *gnom* mutant was cloned using *emb30*, a T-DNA–tagged allele of *gnom. EMB30* encodes a protein similar to the yeast secretory protein Sec7 and may affect cell division, elongation, and adhesion (Shevell et al., 1994). *FUS6* was also cloned by T-DNA tagging; it encodes a novel protein that might be involved in signal transduction (Castle and Meinke, 1994). Recently, more genes involved in Arabidopsis embryo development have been characterized. *AGL15*, an *AGAMOUS*-like gene identified by differential display from Brassica and its Arabidopsis homolog, is expressed uniformly in developing embryos (Heck et al., 1995; Rounsley et al., 1995). AGL15 was recently shown to be localized in embryo nuclei (Perry et al., 1996). *KNOLLE* encodes a syntaxin-related protein and may be involved in cytokinesis (Lukowitz et al., 1996). *STM* is an apical meristem-specific gene (Long et al., 1996) and apparently is the Arabidopsis homolog of the maize gene *KNOTTED1* (*KN1*). *KN1* delineates shoot meristem formation during maize embryogenesis (Smith et al., 1995). *STM* is an excellent embryo development marker, even though its expression is not limited to the embryo. *ATML1* is a novel

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homeodomain gene expressed during early Arabidopsis embryo formation. Expression occurs first in the apical cell, after which the transcripts localize to the protoderm of the 16-cell embryo. *ATML1* transcripts reappear later in the L1 layer of the shoot apical meristem in the mature embryo. Lu et al. (1996) speculated that *ATML1* may be involved in determining morphogenetic boundaries in the early embryo.

Because few genes involved specifically in embryogenesis were available, we decided to isolate additional genes that would serve as molecular markers of early embryo development. Assuming that embryo transcription factors and signal transduction proteins play key roles in establishing embryo structures and patterns, we developed new methods to isolate genes encoding rare and moderately abundant mRNAs expressed in Arabidopsis embryos. Several novel genes have been isolated and analyzed. Here, we describe one of these genes, *PEI1*, which encodes an embryo-specific transcription factor required for the globular- to heart-stage transition.

RESULTS

Isolation of Embryo-Specific Genes by Using Virtual Subtraction

A new approach termed virtual subtraction was developed to isolate tissue-specific genes and genes represented by low-abundance mRNAs from any target tissue. cDNA libraries are displayed at low density so that individual cDNA recombinants can be easily separated. These are screened with cDNA probes made in single-tube random-primed polymerase chain reactions (RP-PCRs) from RNAs that differ from the RNAs used to create cDNA libraries. Hybridized cDNAs represent genes that are expressed in both the target and subtractive tissues; these are "virtually subtracted" from the target libraries and thus are not considered further. Nonhybridizing cDNAs are selected as putative target tissue-specific genes and are further analyzed by one-pass sequencing and RNA expression analysis. Most recently, cDNA libraries are arrayed in 384-well microtiter plates and are then displayed robotically at high density on nylon filters (Nuccio et al., 1997).

We used virtual subtraction to isolate genes expressed in Arabidopsis embryos. A developing seed cDNA library was constructed using poly(A)$^+$ RNA isolated from immature Arabidopsis seeds containing globular- to cotyledon-stage embryos. cDNA clones were screened with mixed cDNA probes prepared by RP-PCR from leaf, root, or silique coat tissues. Abundant seed cDNAs, for example, 12S and 2S seed protein cDNAs, were included in RP-PCR reactions as additional templates to make subtractive probes for subsequent screens. Fifty potential seed-specific cDNA clones were isolated in an initial screen of ~500 clones; these were sequenced from their 5′ termini. The resulting sequences were used in BLAST searches of the GenBank database (Altschul et al., 1990). Several of these cDNA sequences share significant sequence similarities to transcription factors, protein kinases, or protein phosphatases (Table 1).

Thirty of the potential seed-specific cDNAs were selected for RNA gel blot analysis based on their novelty or similarity to other known genes of interest. Four of them, *AtS20*, *AtS29*, *PEI1*, and *AtS478*, were identified as novel seed-specific genes (Table 1). We identified three other genes with important demonstrated or potential biological functions. *AtS212* is identical to the *LEC1* gene isolated by J.J. Harada and colleagues (T. Lotan, M. Ohto, K.M. Yee, M.A.L. West, R. Lo, R.W. Kwong, K. Yamagishi, R.L. Fischer, R.B. Goldberg, and J.J. Harada, submitted manuscript). *LEC1* belongs to a well-known class of transcription factors and plays a key role in regulating late embryonic development (West et al., 1994). *AtS372* is similar to *ABI1* and *ABI2*, which encode homologous protein phosphatases 2C (Leung et al., 1994, 1997). *AtS214* is similar to numerous receptor kinases, including *CLAVATA1* (Clark et al., 1997), and a

Table 1. Novel Arabidopsis Seed cDNAs Identified by Virtual Subtraction

	Expression[a]									
Gene Name	F	L	R	S1	Se	Si	Gene Copy No.[b]	Similar Sequences	Score/P(N)[c]	GenBank Accession No.
AtS20	−	−	−	−	+	+	3	Arabidopsis EST	317/8e-39	T21984
AtS29	−	−	−	−	+	−	1	Anonymous		
PEI1	−	−	−	−	+	−	1	Zn finger protein	76/2e-5	S22126
AtS212	+	+	−	+	+	+	1	*LEC1*		
AtS214	+	+	+	+	+	+	1	Receptor kinase	187/9e-39	U96879
AtS372	+	+	+	+	+	+	1	Protein phosphatase 2C	93/4e-5	P34221
AtS378	−	−	−	−	+	−	1	Anonymous		

[a] Expression data are based on RNA gel blot analysis. F, flowers; L, leaves; R, roots; S1, siliques 1 day after flowering; Se, seeds 2 to 5 days after flowering; Si, developing silique coats.

[b] Gene copy numbers were determined from genomic DNA gel blot analysis.

[c] Score/P(N) indicates the high score and the smallest sum probability, respectively, generated by the BLAST algorithm (Altschul et al., 1990).

receptor-like kinase expressed in embryogenic somatic carrot cells (Schmidt et al., 1997). The expression patterns of these genes, determined by RNA gel blot analysis, are summarized in Table 1. The overall success of virtual subtraction is demonstrated by the isolation of novel rare cDNAs that represent transcription factors and signal transduction proteins; the isolation of genes that correspond to known mutants; and the fact that no cDNAs corresponding to genes like actin or tubulin, which are encoded by moderately abundant mRNAs, were isolated.

PEI1 Encodes a Novel, Embryo-Specific Cys_3His Zinc Finger Protein

PEI1 was selected for further analysis based on its novelty and apparent embryo-specific expression pattern. The results of RNA gel blot analysis are shown in Figure 1A. As illustrated, *PEI1* was only detected in immature seed RNA. *PEI1* transcripts were not detected in vegetative tissues even after exposures of up to 10 days (data not shown). Genomic DNA gel blots (Figure 1B) indicated that *PEI1* is a single-copy gene in the Arabidopsis genome.

The *PEI1* cDNA is 1051 bp long and includes a 15-nucleotide poly(A) tail (Figure 2A). The first ATG codon occurs at position 97, with the longest open reading frame encoding 245 amino acids. The predicted PEI1 protein is highly hydrophilic; among its 245 amino acid residues, 80 are charged residues. Figure 2B depicts the distribution of charged amino acids in the predicted PEI1 protein. Basic residues cluster in the middle region in which three Cys/His motifs are located; in contrast, most of the acidic residues are in the 3′ terminal region. The presence of distinct basic and acidic domains is characteristic of many well-studied transcription factors (Harrison, 1991). In addition, there are several short stretches of basic amino acid residues, for example, KIKR, RRDPRR, and RYRTR, in the middle basic domain that may serve as nuclear localization signals (Abel and Theologis, 1995).

BLAST searches (Altschul et al., 1990) indicated that PEI1 is similar to Cys_3His zinc finger proteins, such as *unkempt* of Drosophila (Mohler et al., 1992), and epidermal growth factors and the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate–induced gene products of mouse, rat, and humans (Gomperts et al., 1990; Heximer and Fordyke, 1993; Varnum et al., 1991). The similarity is restricted to the middle region where the Cys_3His motif is located. On either side of this motif, there is an undefined Cys/His motif. The three motifs are different from one another, except that the five residues at the 3′ end of each motif, CPYAH, CEFAH, and CFFAH, are well conserved.

The middle Cys_3His motif is highly conserved. A BLAST search using only this motif identified >30 similar protein domains. PEI1 is one of only three plant proteins with this motif (Fett-Neto and McClung, 1997; GenBank accession number U90439); however, there are no expression data

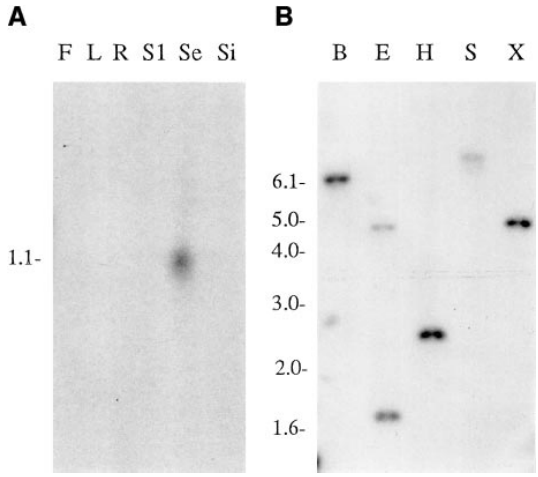

Figure 1. *PEI1* Is an Embryo-Specific, Single-Copy Gene in Arabidopsis.

(A) RNA gel blot analysis of *PEI1* expression. A gel blot containing 10 μg of RNA taken during various stages of Arabidopsis growth and development was hybridized with a radiolabeled *PEI1* probe. F, flower; L, leaf; R, root; S1, silique 1 day after flowering; Se, developing seed; Si, silique with seeds removed.

(B) Genomic DNA gel blot analysis of *PEI1.* DNA gel blots containing 10 μg of Arabidopsis genomic DNA digested with various restriction enzymes were hybridized with a radiolabeled *PEI1* probe. B, BamHI; E, EcoRI; H, HindIII; S, SacI; X, XbaI.

Length markers are given at left in kilobases.

available for the other two. As shown in Figure 2C, both the amino acids and their spacing are highly conserved as $C\text{-}X_{5\text{–}6}G\text{-}X\text{-}C\text{-}X_2\text{-}G\text{-}X_2\text{-}C\text{-}X\text{-}F\text{-}X\text{-}H$, where X is any amino acid. The other two motifs show little homology to any known zinc finger motif. The upstream motif contains two cysteine residues and two histidine residues arranged as $C\text{-}X_5\text{-}H\text{-}X_4\text{-}C\text{-}X_3\text{-}H$. The downstream motif, $C\text{-}X_5\text{-}C\text{-}X_4\text{-}C\text{-}X_3\text{-}H$, contains three cysteine residues and one histidine residue, with spacing different from the middle motif but similar to the upstream motif (Figure 2A).

PEI1 mRNA Is Expressed throughout the Arabidopsis Embryo

In situ hybridization with digoxigenin-labeled RNA probes showed that *PEI1* mRNA is not localized within Arabidopsis embryos. As shown in Figure 3, *PEI1* mRNA is expressed throughout the embryo from early globular stage to cotyledon stage. *PEI1* transcripts were first detected in small patches in early-globular-stage embryos (Figure 3A). Later, *PEI1* transcripts were expressed throughout the embryo but not in the seed coat (Figure 3B). Figures 3C to 3H revealed

A

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1    atgaaatctctg tag ttttttttttgttcctttcttaaatttcgaaagaaagacatttatt
61   aaaccaaaa taa ctctttagatcattgcaaggaaaaatgttgaaaagtgcaagtccaatg
1                                        M  L  K  S  A  S  P  M
121  gcattctacgatatcggagagcagcaatactctactttcgggtacattttaagcaaacct
9    A  F  Y  D  I  G  E  Q  Q  Y  S  T  F  G  Y  I  L  S  K  P
181  gggaacgcaggagcttacgagattgacccttcgatcccaaacatcgacgatgcgatctac
29   G  N  A  G  A  Y  E  I  D  P  S  I  P  N  I  D  D  A  I  Y
241  ggctcagatgagttccgtatgtacgcttacaaaatcaaacggtgtcctcgtactcgtagc
49   G  S  D  E  F  R  M  Y  A  Y  K  I  K  R  C  P  R  T  R  S
301  cacgactggacggagtgtccctacgctcaccgtggcgagaaagccacacgccgtgatcct
69   H  D  W  T  E  C  P  Y  A  H  R  G  E  K  A  T  R  R  D  P
361  cgccgttacacttactgtgcagtcgcatgcccggctttccgaaatggcgcatgccaccgt
89   R  R  Y  T  Y  C  A  V  A  C  P  A  F  R  N  G  A  C  H  R
421  ggcgactcatgcgaattcgcacatggcgtattcgagtactggctccacccggcgcgttac
109  G  D  S  C  E  F  A  H  G  V  F  E  Y  W  L  H  P  A  R  Y
481  cgaacacgcgcatgtaacgccgggaacttgtgtcagaggaaagtgtgtttctttgcccac
129  R  T  R  A  C  N  A  G  N  L  C  Q  R  K  V  C  F  F  A  H
541  gcgccggagcagctaaggcagtctgaaggaaagcacaggtgcaggtacgcatataggccg
149  A  P  E  Q  L  R  Q  S  E  G  K  H  R  C  R  Y  A  Y  R  P
601  gtgagggctagaggtggtggaaacggcgatggagtgacgatgagaatggacgacgagggt
169  V  R  A  R  G  G  G  N  G  D  G  V  T  M  R  M  D  D  E  G
661  tacgacacgtcacggtctccggtgagaagcgggaaagatgatttagatagtaacgaggag
189  Y  D  T  S  R  S  P  V  R  S  G  K  D  D  L  D  S  N  E  E
721  aaggtgttgttgaagtgttggagtcggatgagcattgtggatgatcattatgagccgtcc
209  K  V  L  L  K  C  W  S  R  M  S  I  V  D  D  H  Y  E  P  S
781  gatttggatttggatttgtcacactttgattggatctcagagttggtcgat taa atttgg
229  D  L  D  L  D  L  S  H  F  D  W  I  S  E  L  V  D  *
841  gaaatcaaagcagagaacaaaagaaacccgataaataaagtggattttgttaaaatccac
901  aagatcaagattcaagatgagagatcttgtcatgtatatggtaaatttaattgtaatgat
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1021 tataagtctttgtattaaaaaaaaaaaaaaa
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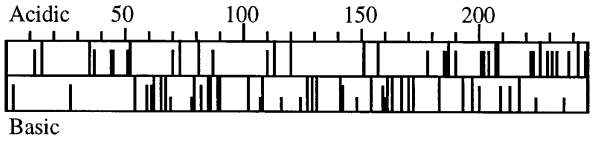
B



C

Pei1	*Arabidopsis*	YCAVA**C**PAFR-NGA**C**HRGDS**C**EFA**H**GVFE
Ytis11p	Yeast	YKTEL**C**ESFTLKGS**C**PYGSK**C**QFA**H**GLGE
ERF1	Human	YKTEL**C**RPFEENGA**C**KYGDK**C**QFA**H**GIHE
TIS11D	Mouse	YKTEL**C**RPFEESGT**C**KYGEK**C**QFA**H**GFHE
TIScc1	*Drosophila*	YKTEL**C**RPFEEAGE**C**KYGEK**C**QFA**H**GSHE
TPA-11	Mouse	YKTEL**C**RTYSESGR**C**RYGAK**C**QFA**H**GLGE
TIS11	Rat	YKTEL**C**RTYSESGR**C**RYGAK**C**QFA**H**GPGE
Unkempt	*Drosophila*	YKSTK**C**NDVQQAGY**C**PRSVF**C**AFA**H**VEPC

Figure 2. *PEI1* Encodes an Unusual Zinc Finger Protein.

(A) *PEI1* cDNA and predicted amino acid sequence. The three Cys/His motifs are underlined, and the conserved cysteine and histidine residues are in boldface. Three in-frame stop codons are also in boldface. The asterisk indicates the functional stop codon.

(B) Schematic representation of acidic and basic residue distribution in the predicted PEI1 protein.

(C) Alignment of representative Cys_3His zinc finger motifs from different species. Only one Cys_3His from each protein was used in the alignment, even though most of them contain multiple motifs. Conserved cysteine and histidine residues are in boldface. GenBank accession numbers of the aligned sequences are *Unkempt*, S22126; ERF1, S33451; TIS11D, P23949; TIScc1, X81194; TPA-11, X14678; TIS11, P47973; Ytis11p, P47977; and Pei1, awaiting number.

maximal expression of *PEI1* from heart stage through cotyledon stage. *PEI1* transcripts were undetectable in embryos just before desiccation (data not shown). Under the same conditions, a seed coat–specific probe, *AtS20*, detected transcripts predominately in the seed coats of embryos from torpedo stage to cotyledon stage (Figure 3H). A β-glucuronidase (*GUS*) probe served as a negative control; as expected, it did not detect any signal (data not shown). These results, along with those shown in Figure 1A, demonstrate that *PEI1* is an embryo-specific gene expressed throughout the embryo from globular stage to cotyledon stage.

Expression of a *PEI1* Antisense Gene Interrupts Embryogenesis at Heart Stage

An antisense gene expression cassette was made by replacing the *GUS* gene in pBI121 (Jefferson et al., 1987) with the *PEI1* cDNA in its antisense orientation; the construct was then introduced into Arabidopsis (Valvekens et al., 1988). Developing seeds of 12 R_1 plants were examined, and five transgenic lines with aberrant seed phenotypes were identified. Four transgenic plant lines, anti-1, anti-2, anti-3, and anti-6, contained white and green seeds in the same siliques. Anti-9 plants contained white, green, and yellow (light green) seeds in the same siliques. These antisense lines were further analyzed by examining developing seeds in the R_3 generation.

Abnormal seeds in siliques of anti-1 plants were white when the normal sibling seeds were at heart stage and had started to turn green; aberrant seeds contained globular- or early-heart-stage embryos (data not shown). To simplify the description of aberrant embryos, the term relative developmental stage (RDS) is used to refer to the normal developmental stage in the same silique, for example, RDS:heart stage. At the time that normal seeds turned green (RDS:cotyledon stage), aberrant sibling seeds remained white and contained enlarged, heartlike embryo structures (Figure 4A). Figure 4B shows the embryos of another anti-1 plant. Siliques containing RDS:late-cotyledon-stage seeds also had white sibling seeds with embryos arrested before heart stage. In some cases, abnormal embryos enlarged to the size of normal embryos, but none of them turned green. It is noteworthy that the basal part of the abnormal white embryos had quite normal embryonic roots; on the other hand, the apical region was severely deformed (Figure 4C). The embryonic phenotypes of anti-2 plants were similar to those of anti-1 plants. One representative anti-2 silique and the embryos dissected from its seeds are shown in Figure 4D.

White anti-3 seeds were smaller than normal green sibling seeds. Some contained embryos at globular stage and occasionally at heart stage. When green seeds contained cotyledon-stage embryos, the white seeds turned brown, shrank, and became flat (Figure 4E). White anti-6 seeds were smaller than sibling green seeds and did not contain enlarged embryos. Most of them did not contain any detectable embryo.

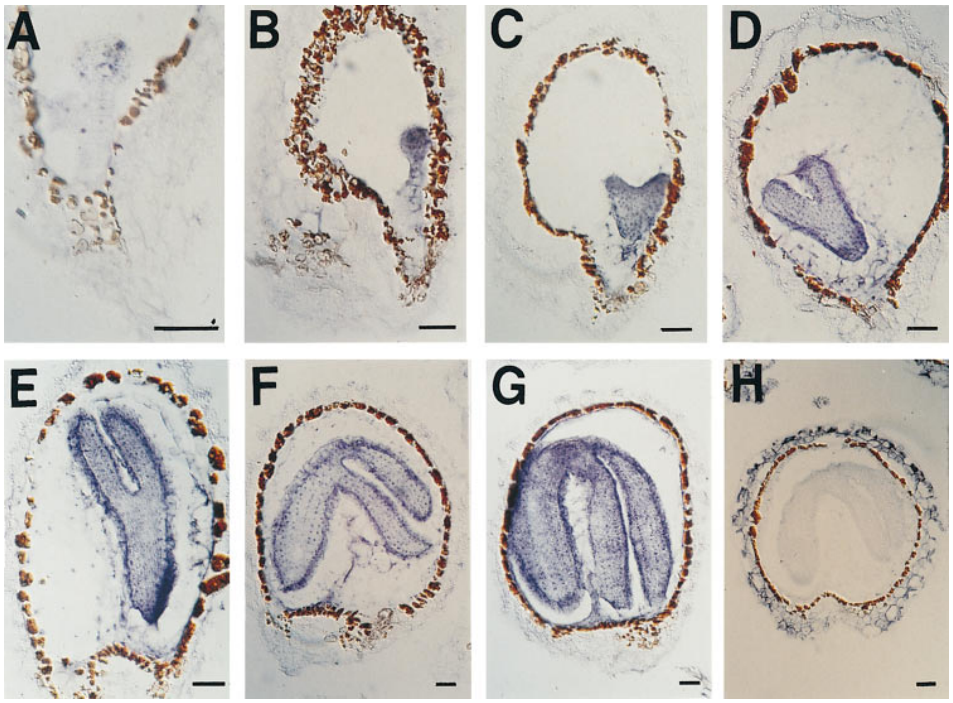


Figure 3. In Situ Hybridization Analysis of *PEI1* Expression.

(A) to **(G)** Embryos at different developmental stages were hybridized with the digoxigenin-labeled *PEI1* antisense RNA probe. In **(A)**, an early-globular-stage embryo with a long suspensor is shown. Globular-, heart-, torpedo-, late-torpedo-, early-cotyledon-, and bent-cotyledon-stage embryos are shown in **(B)** to **(G)**, respectively.
(H) A cotyledon-stage embryo hybridized with a digoxigenin-labeled *AtS20* antisense RNA probe.
Digoxigenin visualization reactions in **(A)** to **(G)** were for 3 days; the digoxigenin visualization reaction in **(H)** was for 24 hr. Bar in **(A)** = 20 μm; bars in **(B)** to **(H)** = 50 μm.

Occasionally, globular embryos were recovered from white anti-6 seeds (see the arrow in Figure 4F). At RDS:late cotyledon stage, most of the sibling white seeds still did not contain detectable embryos (data not shown).

All anti-9 progeny had abnormal seed phenotypes. Their siliques were shorter and wider than normal siliques, which was caused by the abortion of most of their ovules. Aborted ovules were at least two times smaller than were the normal ones (Figure 4G). In addition to white and green seeds, yellow seeds were found in anti-9 siliques. At RDS:late cotyledon stage, the yellow seeds of the siblings contained early-cotyledon-stage embryos, and white seeds contained enlarged globular-stage embryos. An embryo with an enlarged suspensor is indicated by an arrow in Figure 4G. Apparently, embryo development of anti-9 plants was affected to different degrees. White seeds were severely affected, enlarging slightly but not passing heart stage. Yellow seeds were less affected; their embryos eventually developed to maturity.

The major conclusion from the preceding experiments is that aberrant anti-*PEI1* embryos failed to undergo normal embryogenesis at or just before heart stage. They were arrested either with little enlargement or enlargement to a size comparable to that of normal embryos, but none of them turned green. The apical domain was primarily affected; there was no discernible effect on the embryonic root. Although there was some variation in the extent and timing of the anti-*PEI1*–induced morphological perturbations, expression of the anti-*PEI1* gene had a major reproducible effect on embryogenesis, specifically at the globular- to heart-stage transition. Variation in the anti-*PEI1*–induced phenotype probably reflects variation in the level of antisense gene expression.

Embryo Morphology Is Perturbed in Anti-*PEI1* Seeds

White seeds and green seeds at different developmental stages were collected separately from siliques of anti-*PEI1-1* plants and were then prepared for light microscopy. Because it was impossible to distinguish abnormal seeds from normal seeds when both were white, all of the seeds from the same side of a young anti-1 silique were fixed together and sectioned individually later. All of the globular-stage

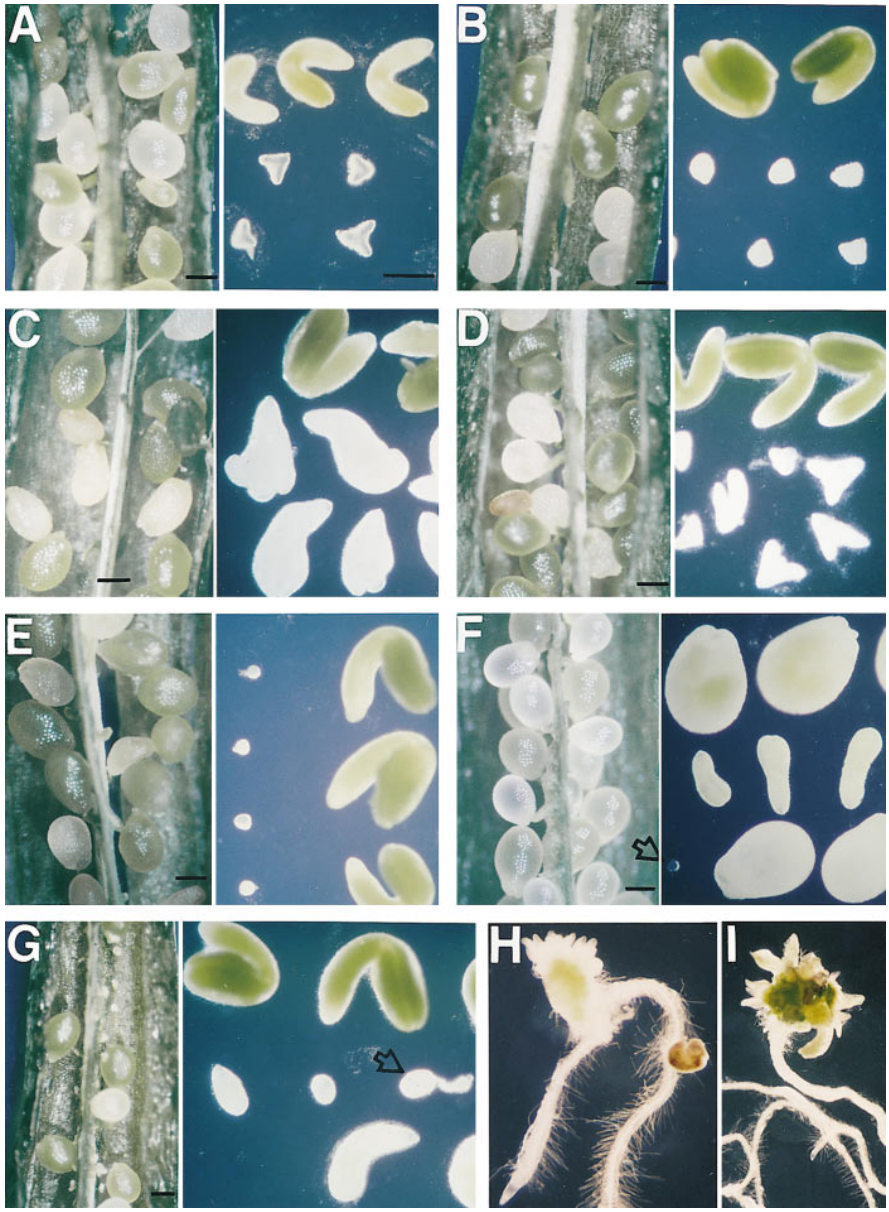


Figure 4. Characteristic Abnormal Embryo Phenotypes of Five *PEI1* Antisense Lines.

Siliques were opened and photographed (left); embryos were then excised from the seeds of the same siliques and photographed (right). In **(A)** to **(D)**, embryos from the green seeds are shown in the top row; embryos from the white seeds are shown in the bottom two rows.
(A) Young anti-1 seeds and embryos.
(B) Seeds and embryos of another anti-1 plant.
(C) Late-stage anti-1 seeds and embryos from the same plant as shown in **(A)**.
(D) Anti-2 seeds and their embryos.
(E) Anti-3 seeds and embryos. Embryos from the white seeds are at left; embryos from the green seeds are at right.
(F) Young anti-6 seeds and embryos. The light green seeds are at top; their embryos are at center; the white seeds and a globular embryo (arrow) are at bottom.
(G) Anti-9 seeds and embryos. Embryos from the green seeds are at top; embryos from the white seeds are at center; embryos from the yellow seeds are at bottom. An embryo with an enlarged suspensor is indicated by the arrow.
(H) Ten-day-old anti-1 abnormal seedling.
(I) One-month-old anti-1 abnormal plantlet.
The same magnification was used for **(A)** to **(I)**; bars = 200 μm.

embryos from the young silique appeared to be normal. Figure 5A shows an early-globular-stage embryo with a long suspensor. Cellular endosperm had not formed at this stage. Instead, there were embryonic cell aggregates attached to the inner seed coat (arrow). In a later-globular-stage embryo, protoderm differentiation was apparent, as depicted in Figure 5B. Once embryo development passed heart stage, normal seeds began to turn green, whereas the abnormal seeds remained white. The green seeds followed all of the normal steps of embryo development to maturity. Heart- and torpedo-stage embryos from green seeds are shown in Figures 5C and 5D, respectively.

Abnormal embryos had variable morphology (Figures 5E to 5L); however, none of them had normal cotyledonary primordia. Aberrant structures (Figure 5E and 5F) were first observed at RDS:heart stage. Aberrant embryos had unorganized cellular proliferation at their apical ends and contained many vacuolated cells. At RDS:torpedo stage (Figure 5D), aberrant sibling embryos exhibited perturbed morphologies (Figures 5G to 5J). Some had protoderm-like cell layers (Figures 5F and 5G), which might be maintained even to maturation stage (Figure 5L). Other aberrant embryos did not have any distinctive structural differentiation (Figures 5I and 5J). RDS:late-cotyledon-stage embryos filled the space left by the degraded endosperm; in contrast, aberrant sibling embryos, which had undefined structures, occupied only a small part of the seed (Figure 5K). In conclusion, aberrant anti-*PEI1* embryos were indistinguishable from normal embryos before heart stage. Later, aberrant embryos were either arrested at heart stage or enlarged to form various abnormal structures. At all stages, aberrant embryos were more highly vacuolated than were their normal siblings.

Even though aberrant embryos failed to form normal embryo structures, they established and maintained polarity. The radicle ends of the deviant embryos retained normal suspensors and thus were less affected than the apical ends (Figures 5E, 5G, and 5H). For example, the youngest abnormal seed sectioned had a late-globular-stage embryo with a normal suspensor (Figure 5E). The apical part of this embryo proliferated at the positions where the normal cotyledons should be. The unimpeded proliferation destroyed the border between the protoderm and its inner tissue. In contrast, the lower half of this embryo, including the long suspensor, appeared to be normal. Similarly, other immature aberrant embryos also possessed suspensors (Figures 5F to 5J), which degraded at the same developmental time as those of normal embryos. Older aberrant embryos with undefined structures still maintained apical–basal polarity (Figure 5L).

Despite aberrant embryo development, endosperm and seed coat development appeared to be normal. Cellular endosperm was always observed in both the aberrant and normal seeds from heart stage to torpedo stage (Figures 5C to 5I). Like normal seeds, endosperm of aberrant seeds also degraded after torpedo stage, even though the abnormal embryos were small and did not need endosperm degradation to provide more space (Figures 5J and 5K).

PEI1 Gene Expression in *PEI1* Antisense Plants

The presence of the transgenic *PEI1* antisense gene in the five antisense lines was verified by DNA gel blot analysis. Because HindIII does not cut *PEI1* cDNA internally, any hybridization band generated by HindIII digestion represents one copy of the *PEI1* gene, either sense or antisense. As shown in Figure 6A, both anti-1 and anti-2 plants had only one copy of the *PEI1* antisense gene represented by the extra band (lanes 1 and 2) in addition to the endogenous *PEI1* gene band, as was detected in wild-type plants (lane wt). Anti-3 plants had three copies of the *PEI1* antisense gene, as indicated by the three additional hybridization bands (lane 3). Anti-6 plants also had three extra hybridizing bands (lane 6). Two of these bands were more intense than the endogenous *PEI1* gene band; thus, each might represent more than one copy of the transgene. Anti-9 plants had two copies of the transgene (lane 9).

Because the cauliflower mosaic virus 35S promoter used to drive *PEI1* antisense gene expression is nominally a constitutive promoter especially active in leaves and seedlings, RNA gel blotting was done using RNA from leaves of different antisense plants to evaluate anti-*PEI1* expression. Seedling or flower RNA was also analyzed for some lines. Because *PEI1* is only expressed in developing seeds of wild-type Arabidopsis plants, any signal detected by the *PEI1* cDNA probe in tissues other than developing seeds of the transgenic plants represents *PEI1* antisense transcripts. As shown in Figure 6B, all of the antisense plants expressed the *PEI1* antisense gene. The expression levels did not appear to correlate with the copy numbers of the antisense gene, that is, antisense plants with more copies did not necessarily have higher expression levels. Anti-9 plants (lane 9L), which had two copies of the antisense gene, had higher expression than did anti-3 or anti-6 plants, which had three or even more copies (lanes 3L and 6L). In addition to the major transcript, anti-6 plants had a larger minor transcript. This larger transcript may have been the result of leaky transcriptional termination by the nopaline synthase terminator. As expected, the antisense *PEI1* gene was also expressed in seedlings of anti-1 and anti-2 plants (lanes 1S and 2S) and in anti-9 flowers (lane 9F). The anti-1 developing green seed RNA (lane G) and the wild-type seedling RNA (lane wtS) were used as positive and negative controls, respectively. The preceding showed that *PEI1* antisense transcripts were stable in different vegetative tissues, and as expected, *PEI1* antisense transcripts did not affect the normal development of any organ other than the seed.

We then asked whether the *PEI1* antisense transcripts interacted with the sense transcripts in seeds in which *PEI1* is normally expressed. If the antisense transcripts triggered *PEI1* mRNA degradation in the abnormal seeds, abnormal seeds should have fewer *PEI1* transcripts than normal sibling seeds. Figure 7A depicts results of hybridization of *PEI1* with RNA gel blots containing RNA from developing anti-1 white and green seeds from the same siliques. *PEI1* transcripts in

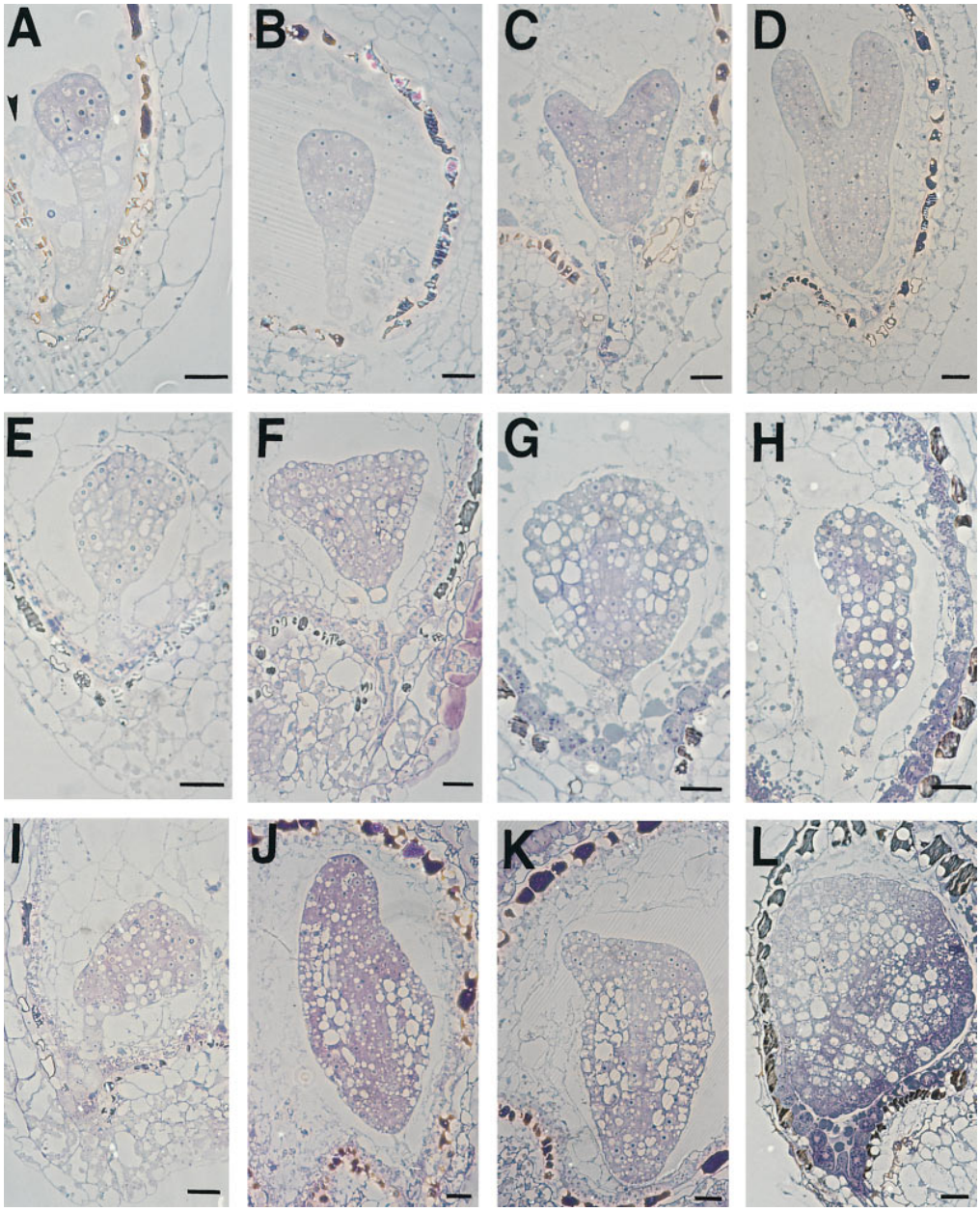


Figure 5. Histological Analysis of *PEI1* Anti-1 Seeds.

PEI1 anti-1 developing green and white seeds were sectioned separately. **(A)** to **(D)** show green seeds. **(E)** to **(L)** show white seeds. The RDS of the abnormal embryos was determined from normal embryos in the same siliques.
(A) Early-globular-stage embryo. Arrowhead indicates embryonic cell aggregates.
(B) Late-globular-stage embryo.
(C) Heart-stage embryo.
(D) Torpedo-stage embryo.
(E) Abnormal embryo at RDS:early heart stage.
(F) Abnormal embryo at RDS:heart stage.
(G) and **(H)** Abnormal embryos at RDS:torpedo stage.
(I) and **(J)** Abnormal embryos at RDS:early cotyledon stage.
(K) Abnormal embryo at RDS:late cotyledon stage.
(L) Mature abnormal embryo.
Bars in **(A)** to **(L)** = 20 μm.

white seeds were in approximately the same abundance as in green seeds, suggesting that the presence of anti-*PEI1* did not result in accelerated *PEI1* mRNA turnover.

Given that *PEI1* mRNA degradation was apparently not a factor in the effect of anti-*PEI1*, levels of PEI1 protein in embryos were determined. A polyclonal antibody against PEI1 was raised using recombinant PEI1 protein. As depicted in Figure 7B, this antibody recognized a single band on gel blots containing total proteins extracted from developing seeds. No signal was detected with protein from flowers, leaves, silique coats, or stems. These results verified the RNA gel blot hybridization result (Figure 1)—*PEI1* is a seed-specific gene. Protein gel blot analysis also revealed that PEI1 is enriched in nuclear extracts, because a stronger signal was obtained with 5 μg of nuclear extract prepared from developing Arabidopsis siliques when compared with the signal obtained with 30 μg of total Arabidopsis cellular proteins (cf. Figure 7B, lanes Se, G, W, and NE). The recombinant PEI1 protein (Figure 7B, pET) contains a 5-kD leader peptide translated from the pET30 vector sequence; therefore, the positive control band migrated more slowly than did the non-recombinant protein (Figure 7B, lanes Se, G, W, and NE). Of the three seed protein lanes, the PEI1 protein in the anti-1 white seeds (lane W) was 28% of the protein in the green seeds (lane G), even though the white and green seeds were collected from the same siliques. Wild-type seeds (lane Se) had 25% more PEI1 than did the anti-1 green seeds. These results suggest that PEI1 expression was attenuated in aberrant seeds.

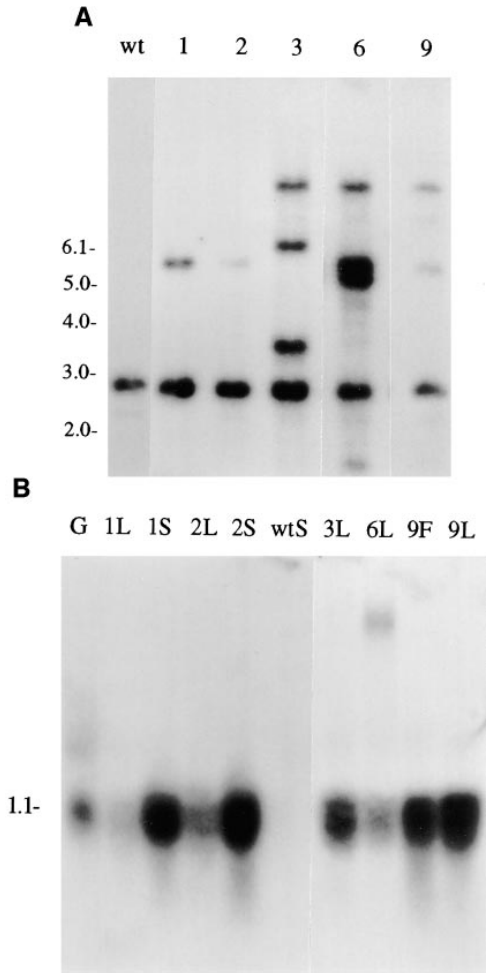


Figure 6. The *PEI1* Antisense Gene Is Expressed in All of the *PEI1* Antisense Lines.

(A) DNA gel blot analysis of the *PEI1* antisense plants. A gel blot containing *PEI1* antisense plant genomic DNA digested with HindIII was hybridized with the full-length *PEI1* cDNA probe. Lane wt, genomic DNA from untransformed Arabidopsis; lane 1, anti-1; lane 2, anti-2; lane 3, anti-3; lane 6, anti-6; and lane 9, anti-9. Fragment lengths are given at left in kilobases.

(B) *PEI1* antisense gene expression in *PEI1* antisense lines. Gel blots containing RNA extracted from different *PEI1* antisense plants were hybridized with the full-length *PEI1* cDNA probe. G, RNA from developing anti-1 green seeds; 1L, anti-1 leaves; 1S, anti-1 seedlings; 2L, anti-2 leaves; 2S, anti-2 seedlings; wtS, wild-type seedlings; 3L, anti-3 leaves; 6L, anti-6 leaves; 9F, anti-9 flowers; and 9L, anti-9 leaves. mRNA length is given at left in kilobases.

Genes Involved in Photomorphogenesis Are Not Expressed in Anti-*PEI1-1* Seeds

The preceding data suggest that PEI1 might be a transcription factor playing an indispensable role in the formation of heart- and later-stage embryos. If so, the deficiency of PEI1 in abnormal seeds might affect the expression of genes downstream of *PEI1.* Three classes of genes—constitutive, seed specific, and chloroplast specific—were selected so that their expression in white and green seeds from sibling anti-1 plants could be compared; the results of these experiments are depicted in Figure 8. Constitutive genes, such as the ribosome S17 protein gene (Figure 8) and a tubulin gene (data not shown), each had similar expression levels in both green and white seeds, indicating that the basic gene expression machinery was not affected in *PEI1* antisense plants. Seed-specific genes, such as 2S albumin, 12S cruciferin, and oleosin genes, were all expressed in white seeds, but at levels somewhat lower than in the green seeds (data not shown). Similarly, a novel embryo-specific gene, *AtS478* (Table 1), was expressed, but at slightly lower levels in abnormal seeds (Figure 8). Thus, the expression of these seed-specific genes was attenuated rather than silenced in *PEI1* antisense plants.

In contrast, several genes known to be involved in chlorophyll synthesis or photosynthesis were turned off in white anti-*PEI1-1* seeds. The results are depicted in Figure 8. A protochlorophyllide oxidoreductase (POR) gene, whose product catalyzes a critical step of chlorophyll synthesis (Reinbothe et al., 1996), was completely turned off in aberrant anti-*PEI1-1* seeds (*POR*, lane W). Likewise, the ribulose-1,5-bisphosphate

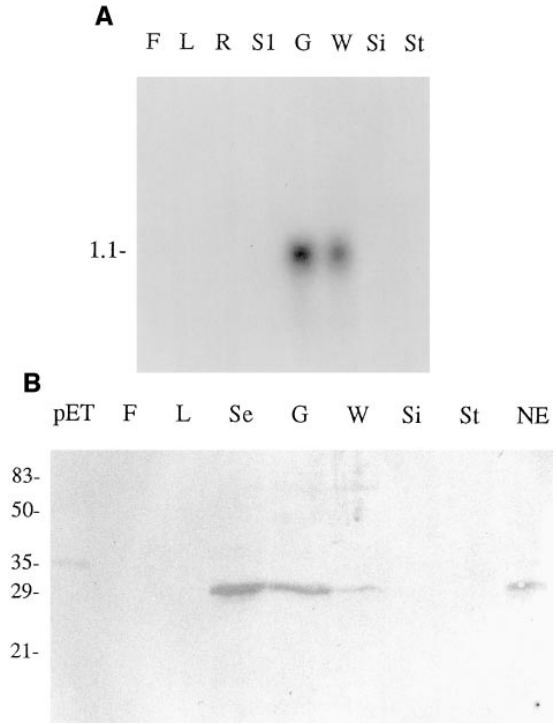


Figure 7. Comparison of *PEI1* Transcripts and PEI1 Protein Levels in Anti-1 Abnormal White Seeds and Normal Green Seeds.

(A) RNA gel blot analysis of *PEI1* transcripts. The RNA gel blot was hybridized with the full-length *PEI1* cDNA probe. All of the tissues except seeds were from wild-type plants. F, flowers; L, leaves; R, roots; S1, siliques 1 day after flowering; G, *PEI1* anti-1 developing green seeds; W, anti-1 white seeds; Si, developing silique coats; St, stems.

(B) Protein gel blot analysis of wild-type and anti-*PEI1* transgenic plants. The gel blot was first incubated with anti-PEI1 antibody. The secondary antibody was a goat anti–rabbit Fab′ labeled with iodine-125. All of the tissues except green and white seeds were from wild-type plants. pET, 10 ng of recombinant PEI1 protein expressed in *E. coli* with the pET-30a expression vector (see Methods); F, flowers; L, leaves; Se, developing seeds; G, green seeds from anti-1 plants; W, white seeds from anti-1 plants; Si, developing silique coats; St, stems; NE, nuclear extracts. Lanes F, L, Se, G, W, Si, and St contain 30 μg of total proteins; lane NE contains 5 μg of nuclear extracts prepared from developing siliques.

Length markers are indicated at left in kilodaltons.

carboxylase small subunit gene (*rbcS*, lane W) and a chlorophyll *a*/*b* binding protein gene (*Cab*, lane W) were not detectable in abnormal seeds. On the other hand, the chloroplast 16S ribosomal RNA gene was expressed in both abnormal seeds (lane W) and green seeds (lane G). This indicated that some genes required for chloroplast development were still active in the abnormal seeds, even though *PEI1* gene repression severely affected embryonic photomorphogenesis.

Rescue of Anti-*PEI1-1* Immature Seeds

We attempted to rescue aberrant anti-1 embryos by tissue culture. White abnormal seeds and normal green seeds of anti-1 plants were plated on media formulated for Arabidopsis embryo culture (Wu et al., 1992). Green seeds containing cotyledon-stage embryos germinated in ~1 week. The embryonic roots elongated quickly and generated lateral roots; the cotyledons expanded and turned green (data not shown). Aberrant embryos (RDS:cotyledon stage) germi-

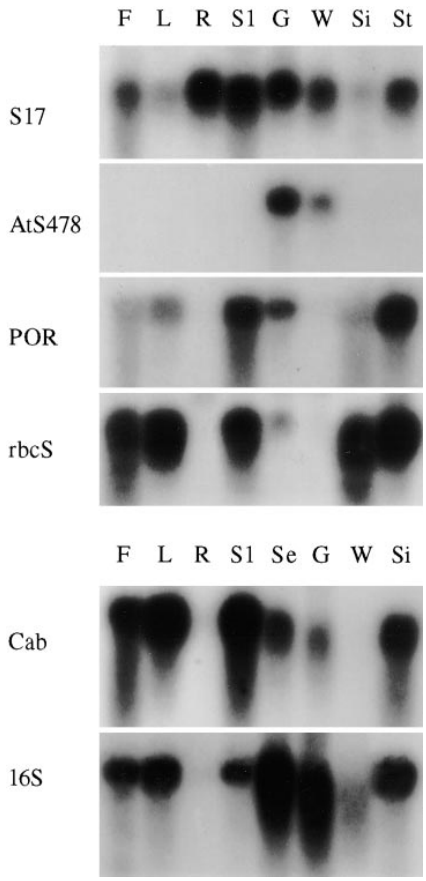


Figure 8. Gene Expression in Normal and Abnormal *PEI1* Anti-1 Developing Seeds.

RNA gel blots were hybridized with probes derived from different Arabidopsis genes. Lanes contain RNA from flowers (F), leaves (L), roots (R), siliques 1 day after flowering (S1), developing seeds (Se), anti-1 developing green (G) and white (W) seeds, developing silique coats (Si), and stems (St). All of the tissues, except for green and white seeds, were from wild-type plants. The probes are the ribosome S17 protein gene (*S17*), a novel embryo-specific gene (*AtS478*), protochlorophyllide oxidoreductase gene (*POR*), ribulose-1,5-bisphosphate carboxylase small subunit gene (*rbcS*), chlorophyll *a*/*b* binding protein gene (*Cab*), and the chloroplast 16S ribosomal RNA gene (16S).

nated ~3 days later than did the green seeds. The embryonic roots elongated and formed lateral roots, as did the normal seeds. However, the apical organs of the newly formed seedlings were abnormal. Some had two cotyledon-like structures; some had multiple leaf primordia (Figure 4H); some did not have any cotyledon-like or leaflike structure. After being transferred to germination media, the apical parts of the abnormal seedlings generated more leaflike structures and turned green, especially in the center of the leaf cluster (Figure 4I). The roots continued to grow and branch, forming an apparently normal root system. However, no normal plant was ever formed directly from the abnormal seeds.

Because germinated aberrant seeds developed normal root systems, we thought that it might be possible to generate plantlets from roots if PEI1 were required only for embryogenesis but not for vegetative shoot development. Roots derived from aberrant seeds were first cultured on Murashige and Skoog media (Gibco BRL) and then grown in Gamborg's B_5 liquid media (Gibco BRL). Subsequent callus induction and plant regeneration procedures were similar to those used for root transformation (Valvekens et al., 1988). Callus was readily induced from these roots and was able to differentiate. Several green plantlets were recovered. One plantlet developed roots and shoots and was transferred to soil. Developing seeds of this regenerated plant had exactly the same abnormal phenotype as did anti-*PEI1-1* plants. Other regenerated plants also set seeds. These results suggest that PEI1 may be required for development of the apical domain in the embryo and is dispensable elsewhere in the embryo, for example, in roots.

DISCUSSION

Virtual Subtraction: A Robust Approach to Isolate Novel Genes

Virtual subtraction, unlike differential screening, uses one cDNA probe and screens one set of filters. All unhybridized clones are potential tissue-specific target genes. In some respects, virtual subtraction resembles cold-plaque screening (Hodge et al., 1992); however, there are major differences. In particular, high-mass, high–specific activity RP-PCR probes from heterologous tissues are used to probe the target cDNA library. This, combined with robotic preparation of high-density library filter arrays for hybridization, allows rapid analysis of the dynamics of mRNA expression patterns of genes in the target library as well as isolation of low-abundance, constitutive, and tissue-specific genes.

The success of virtual subtraction depends on the hybridization efficiency of the subtractive tissue cDNA probe. A single-tube RP-PCR was used to produce driver quantities of high–specific activity subtractive cDNA probes, starting with as little as 20 ng of cDNA template. Li (1997) showed that RP-PCR probes were two- to 2.5-fold more sensitive in cDNA filter screens when compared with screens with first-strand cDNA probes (Flytzanis et al., 1982; Wilde et al., 1988).

A pilot virtual subtraction screen of ~500 cDNA clones from a developing seed cDNA library yielded 50 candidate seed-specifc cDNAs; of these, four novel seed-specific genes, including *PEI1*, were isolated (Table 1). Although this virtual subtraction screen was not exhaustive, it successfully identified three important genes of known or potential function. *AtS212* is identical to the *LEC1* gene isolated by J.J. Harada and colleagues (T. Lotan, M. Ohto, K.M. Yee, M.A.L. West, R. Lo, R.W. Kwong, K. Yamagishi, R.L. Fischer, K.D. Goldberg, and J.J. Harada, submitted manuscript). *LEC1* is a cardinal transcription factor that integrates several specific aspects of embryonic development (West et al., 1994). *AtS372* is similar but not identical to *ABI1* and *ABI2*, which encode homologous protein phosphatases 2C (Leung et al., 1994, 1997). *AtS214* is similar to a number of receptor kinases, including *CLAVATA1* (Clark et al., 1997). It is also noteworthy that no cDNAs were isolated that correspond to genes such as actin or tubulin, which are encoded by moderately abundant mRNAs.

Expression of a *PEI1* Antisense Gene Induces Aberrant Embryo Phenotypes

To date, antisense and cosuppression techniques are the major approaches to studying the function of previously cloned genes in plants. Antisense gene expression has been extensively used to study gene function (Oeller et al., 1991; Van der Meer et al., 1992; Muschietti et al., 1994), and there are several examples in which stable transgenic antisense genes have been used to generate characteristic mutant phenotypes or to affect the functions of specific genes (Erickson, 1993; Cheung et al., 1995).

Expression of anti-*PEI1* resulted in a range of phenotypes. Anti-1, anti-3, anti-6, and anti-9 plants have their own characteristic embryo phenotypes. Each line can be readily distinguished from the others. Because *PEI1* is exclusively expressed in embryos, it was not surprising that all of the abnormal phenotypes were observed only in embryos. Furthermore, the various phenotypes have at least two features in common. First, these mutants cannot form normal heart-stage embryos. The anti-3, anti-6, and anti-9 white abnormal seeds simply cannot develop beyond heart stage. The anti-1 and anti-2 white abnormal embryos seem to have the ability to form heart-stage embryos, but these heartlike structures have perturbed cellular organization, as shown in Figure 5. Second, abnormal embryos do not turn green during embryogenesis. The distinctive phenotypic differences of the multiple antisense lines demonstrate that *PEI1* gene function is disrupted by the *PEI1* antisense gene to different degrees in these lines, most likely because of slight variations

in the level of anti-*PEI1* expression at critical times in embryogenesis.

The observations that anti-*PEI1* embryos fail to develop cotyledons and yet have apparently normal roots and that, in culture, aberrant seeds developed normal root systems but not leaves suggest that PEI1 may be required for development of the apical domain in the embryo and is dispensable elsewhere in the embryo, for example, in roots. If this is the case, additional localization factors must be involved because *PEI1* appears to be expressed throughout the embryo (Figure 3). It is possible that PEI1 is involved in shoot apical meristem or cotyledon formation, but it is premature to speculate what role this might be. However, this does present an interesting and testable hypothesis that is being addressed by examining PEI1 expression in mutant backgrounds that affect shoot apical meristem and cotyledon formation.

Although the expression of the *PEI1* antisense gene was verified by RNA gel blot hybridization, the behavior of the foreign transcripts is not known. How the antisense transcripts interact with the endogenous sense transcripts is not understood. Thus, it is premature to speculate on a correlation between the abnormal phenotypes and *PEI1* antisense gene copy numbers. Although it was reported that multiple copies of antisense genes are required to block the ethylene biosynthetic pathway in tomato (Theologis et al., 1993), transgenic plants containing only one copy of the *PEI1* antisense gene showed distinctive embryo-defective phenotypes. Threshold levels of antisense gene expression might be required to block the function of an endogenous gene. However, the expression levels may not be proportional to the copy number of the gene. More copies do not necessarily mean higher expression levels. Too many copies may result in cosuppression, turning off antisense gene expression as well (Van Blokland et al., 1994). This may explain why three or more copies of the *PEI1* antisense gene in anti-3 and anti-6 plants did not perform better than one copy in anti-1 and anti-2 plants.

PEI1 Encodes an Unusual Cys_3His Zinc Finger Protein Gene

Zinc finger proteins are diverse compared with other DNA binding proteins. They can be divided into different classes according to the numbers of their conserved cysteine and histidine residues and the spacing between these conserved residues (Evans and Hollenberg, 1988; Harrison, 1991). Different types of plant zinc finger proteins have also been identified, such as the C_2H_2 finger (Takastsuji et al., 1992, 1994; Tague and Goodman, 1995), ring finger (Deng et al., 1992; von Arnim and Deng, 1993), GATA finger (Daniel-Vedele and Caboche, 1993; Putterill et al., 1995), and LIM finger (Sanchez-Garcia and Rabbitts, 1994). However, to our knowledge, the Cys_3His zinc finger found in many epidermal growth factor–responding proteins (Gomperts et al., 1990; Heximer and Forsdyke, 1993; Varnum et al., 1991) and a Drosophila early embryo-specific protein unkempt (Mohler et al., 1992) has only been described in two other plant proteins (Fett-Neto and McClung, 1997; GenBank accession number U90439) in addition to PEI1. Both the key residues and spacing of the PEI1 middle zinc finger are well conserved (Figure 2C). It is not known whether the Cys_3His zinc finger can bind DNA specifically or whether additional sequences are required for DNA binding. However, we used a random binding site selection method to show that recombinant PEI1 protein purified from *Escherichia coli* can bind to specific DNA sequences (Z. Li, S.Y. Kim, and T.L. Thomas, manuscript in preparation). This DNA binding activity supports the prediction that PEI1 is an embryo-specific transcription factor.

Defective Photomorphogenesis in Aberrant Anti-*PEI1* Embryos: A Secondary Effect of Embryogenesis Arrest

Arabidopsis embryos younger than heart stage are white. After heart stage, embryos accumulate pigments and change color from light green at late torpedo stage to dark green at late cotyledon stage. During maturation and desiccation, embryos lose their green color and again turn white. Embryonic photomorphogenesis in Arabidopsis has not been studied extensively. The lack of expression of photomorphogenesis marker genes in aberrant white anti-*PEI1* seeds suggests that PEI1 function is related to embryonic photomorphogenesis. Whether PEI1 is directly involved in regulating these genes is an open question. A previous study of chlorophyll *a*/*b* binding protein gene expression suggested that embryo development control mechanisms supersede light regulatory signals during soybean embryo development (Chang and Walling, 1992). Considering that embryo development in anti-3, anti-6, and anti-9 plants is arrested before heart stage when even normal embryos have not yet started to turn green, it is likely that the arrest of embryo development caused by *PEI1* antisense gene expression results in defective embryonic photomorphogenesis. In addition, the ability of rescued aberrant anti-*PEI1* seeds to germinate and form seedlings with green leaflike structures suggests that the photomorphogenesis machinery in white, aberrant anti-*PEI1* embryos is retained and that the effect of anti-*PEI1* on photomorphogenesis is secondary to embryogenesis arrest.

METHODS

RNA Isolation, cDNA Synthesis, and cDNA Library Construction

Plants (*Arabidopsis thaliana* ecotype Landsberg *erecta*) were grown under continuous illumination in a vermiculite/soil mixture at ambient temperature (22°C). Siliques were dissected 2 to 5 days after flowering to collect immature seeds and silique coats separately. Root tissue was derived from elongated roots of seedlings grown in Gamborg's B5 liquid media (Gibco BRL, Gaithersburg, MD). Flowers, stems, and leaves were also collected. Total RNA was isolated after

a phenol–SDS extraction and LiCl precipitation protocol (Ausubel et al., 1990). Poly(A)$^+$ RNA was isolated using oligo(dT) column chromatography, according to the manufacturer's protocols (Stratagene, La Jolla, CA). RNA minipreps were performed using the RNAeasy plant total RNA purification kit (Qiagen, Chatsworth, CA).

Flower, leaf, root, silique coat, and immature seed cDNAs were synthesized from their poly(A)$^+$ RNA, according to the manufacturers' protocols (Gibco BRL; Stratagene). cDNAs >300 bp were enriched by Sephacryl S-400 column chromatography (Stratagene). The developing seed and root cDNA libraries were constructed from 5 μg of poly(A)$^+$ RNA, respectively, using a ZAP-cDNA synthesis kit (Stratagene). The cDNAs were directionally cloned into the EcoRI and XhoI sites of pBluescript SK− (Stratagene) in the λ ZAPII vector. Nonrecombinant backgrounds for the root and the developing seed cDNA libraries were 2.5 and 1.9%, respectively.

Random-Primed Polymerase Chain Reaction

Twenty nanograms of double-stranded cDNAs was used as templates in 25-μL random-primed polymerase chain reaction (RP-PCR) labeling reactions containing 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 2 mM $MgCl_2$, 10 μM random hexamers (Boehringer Mannheim, Indianapolis, IN), 200 μM each dCTP, dGTP, and dTTP, 50 μM cold dATP, 8 μM α-^{32}P–dATP, 800 Ci/mmol (Du Pont, Boston, MA), and 5 units of Taq DNA polymerase (Promega, Madison, WI). The following program was used for most RP-PCRs: 95°C for 5 min and then 40 cycles of 95°C for 30 sec, 18°C for 1 sec, ramp to 30°C at a rate of 0.1°C/sec, and then 72°C for 1 min. Sephadex G-50 quick spin columns (Boehringer Mannheim) were used to remove unincorporated deoxynucleotide triphosphates.

Virtual Subtraction

A fraction of the developing seed cDNA phage library was converted to plasmid cDNA library by mass excision (Stratagene). Plasmid DNAs were prepared by the boiling mini-prep method (Holmes and Quigley, 1981) from randomly selected clones. cDNA inserts were excised by EcoRI and XhoI double digestion and resolved on 0.8% agarose gels. Denatured DNA was transferred to nylon membranes (Micron Separation Inc., Westborough, MA) in 10 × SSC (1 × SSC is 0.15M NaCl and 0.015 M sodium citrate), and the resulting filters were hybridized with RP-PCR cDNA probes (Ausubel et al., 1990).

RNA and DNA Gel Blot Analyses

Ten micrograms of total RNA extracted from different sources was run on 1.2% agarose gels containing 7% formaldehyde in 1 × Mops buffer (20 mM 3-[*N*-morpholino]propanesulfonic acid, 5 mM sodium acetate, and 1 mM EDTA, pH 6.0) and transferred to nylon filters (Micron Separations Inc.) in 10 × SSC. Blots were hybridized with probes prepared from gel-purified cDNA templates in 50% formamide, 5 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM sodium phosphate, and 1 mM EDTA, pH 7.4), 1 × Denhardt's solution (0.02% Ficoll, 0.02% PVP, and 0.02% BSA), 0.1% SDS, and 100 μg/mL denatured salmon sperm DNA at 42°C for 24 hr. Radioactive probes were prepared by a modified random-primed DNA labeling method (Feinberg and Vogelstein, 1983). Filters were washed in 600 mM NaCl, 80 mM Tris-HCl, pH 7.0, 4 mM EDTA, 12.5 mM phosphate buffer, pH 6.8, and 0.2% SDS at 65°C for 20 min, followed by another 20 min at 65°C in 150 mM NaCl, 20 mM Tris, pH 7.0, 2 mM EDTA, 12.5 mM phosphate buffer, pH 6.8, and 0.2% SDS. Hybridization was detected by autoradiography and quantified using a PhosphorImager (Fuji, Stamford, CT).

For gel blot hybridization analysis, genomic DNA was prepared from whole plants using the hexadecyltrimethylammonuium bromide (Sigma) method (Ausubel et al., 1990). Ten micrograms of DNA was digested with different restriction enzymes and resolved on 0.7% agarose gels. DNA was transferred to Hybond N$^+$ membranes (Amersham, Arlington Heights, IL) with 0.4 N NaOH. Hybridization conditions were identical to those used for virtual subtraction.

Sequence Analysis

Mini-prep plasmid DNA was sequenced using the SequiTherm cycle sequencing kit (Epicentre, Madison, WI). Sequences were analyzed locally using GeneWorks 2.3 (IntelliGenetics, Inc., Campbell, CA). Database searches were done using the BLAST algorithm (Altschul et al., 1990), and the results were subsequently analyzed using various applications in the BCM search launcher (http://kiwi.imgen.bcm.tmc.edu).

In Situ Hybridization

Developing seeds were collected from siliques 2 to 5 days after flowering and fixed at 4°C overnight with 4% formaldehyde and 0.5% glutaraldehyde in 100 mM phosphate buffer, pH 7.0. Fixed seeds were embedded in Paraplast (Sigma) and sectioned at 8 μm. Antisense RNA probes were labeled in vitro from cDNA inserts in pBluescript SK− with digoxigenin–UTP by T7 RNA polymerase using a digoxigenin RNA labeling kit (Boehringer Mannheim). RNA probes were hydrolyzed to 200 nucleotides average length by mild alkali treatment (Cox and Goldberg, 1988). Slides were pretreated and hybridized with 300 ng/mL probes at 55°C overnight in hybridization solution containing 50% formamide (Leitch et al., 1994). Hybridized probes were detected using an anti-digoxigenin antibody conjugated with alkaline phosphatase that catalyzed the reaction between 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Boehringer Mannheim) to form stable purple precipitates. Photographs were taken using Zeiss Axiophot (Jena, Germany) and Olympus BH-2 (Melville, NY) compound microscopes with differential interference contrast optics.

Transgenic Plant Analysis

The full-length *PEI1* cDNA in pBluescript SK− was excised with XhoI and SacI and subcloned into the HindIII and SacI sites in the pBI121 binary vector (Clontech, Palo Alto, CA). The XhoI and HindIII cohesive ends were filled in with the Klenow fragment of DNA polymerase I before ligation. This antisense construct was transferred into *Agrobacterium tumefaciens* LBA4404, which was then used to transform Arabidopsis roots (Valvekens et al., 1988). Regenerated transgenic plants were grown on kanamycin containing Murashige and Skoog media (Gibco BRL) in Magenta boxes; 12 independent transgenic lines were recovered. R_1 and R_2 transgenic plants were grown in vermiculite/soil mixture and assayed for their phenotypes. Seeds and the embryos dissected from them were photographed using an

Olympus SZH10 dissecting microscope with bright- or dark-field illumination.

Histological Analysis

White aberrant seeds and green normal seeds of anti-1 plants were fixed separately, as described for in situ hybridization, and embedded in London Resin white (Electron Microscopy Sciences, Fort Washington, PA) (VandenBosch et al., 1994). Semisections were cut with an ultracut E microtome (Reichert-Jung, Deerfield, IL) at 500 nm or 1 μm and stained with toluidine blue. Photographs were taken using Zeiss Axiophot or Olympus BH-2 microscopes.

Expression of *PEI1* cDNA in *Escherichia coli*

The *PEI1* coding sequence and its 3′ untranslated region including the poly(A) tail were amplified using the T7 primer 5′-CGGGATATC-ACTCAGCATAATG-3′ and the primer 5′-CGGGATCCATGGTGA-AAAGTGCAAGTCC-3′ complementary to the 5′ end of the cDNA coding region. The underlined sequence is an NcoI site at which the last G is a mismatch with the T of the cDNA at the corresponding position. Amplified cDNA was cloned into the NcoI and XhoI sites of the expression vector pET-30a(+) (Novagen, Madison, WI) that was then introduced into BL21(DE3) host cells. The PEI1 protein was induced and then purified using nickel affinity chromatography, according to the manufacturer's protocol (Novagen). The protein was further purified by SDS-PAGE and used to raise anti-PEI1 antibody in rabbits by Veterinary Clinical Resources (Elgin, TX).

Protein Gel Blot Analysis

Total protein was extracted in buffer containing 50 mM sodium phosphate, pH 7.0, 10 mM EDTA, 10 mM β-mercaptoethanol, 0.1% Triton X-100, and 0.1% sodium lauryl sarcosine. Silique nuclear extracts were prepared from developing Arabidopsis siliques according to a published protocol (Kim et al., 1994). Proteins were separated by SDS-PAGE and transferred to nitrocellulose (Micron Separations Inc.) in 12 mM Tris base, 96 mM glycine, pH 8.3, and 20% methanol. The blots were blocked with 2% BSA in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20). Anti-PEI1 antiserum (1:3000) in the blocking solution was used as the primary antibody. The secondary antibody was either anti–rabbit IgG (goat) conjugated with alkaline phosphatase (Sigma) or anti–rabbit Fab′ (goat) labeled with iodine-125 (Amersham); the former was developed using the BCIP/NBT system (5-bromo-4-chloroindolyl-phosphate; Boehringer Mannheim) and the latter by autoradiography. Quantification of the bands was done using the MacBAS 2.1 program (Fuji, Stamford, CT).

Embryo Rescue

Developing seeds were dissected from their siliques and placed on Gamborg's B5 media supplemented with 5% sucrose, 400 mg/L glutamine, 0.01 mg/L indoleacetic acid, and 0.005 mg/L kinetin (Wu et al., 1992) and cultured at 25°C with 24-hr illumination. Germinated embryos were transferred to Murashige and Skoog media supplemented with 1% sucrose for further growth.

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