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The Regulator of *MAT2* (ROM2) Protein Binds to Early Maturation Promoters and Represses PvALF-Activated Transcription

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The regulation of maturation (*MAT*)- and late embryogenesis (*LEA*)-specific gene expression in dicots involves factors related to ABI3, a seed-specific component of the abscisic acid signal transduction pathways from Arabidopsis. In French bean (*Phaseolus vulgaris*), the ABI3-like factor, PvALF, activates transcription from *MAT* promoters of phytohemagglutinin (*DLEC2*) and β-phaseolin (*PHSβ*) genes. We describe the regulator of *MAT2* (ROM2) as a basic leucine zipper (bZIP) DNA binding protein that recognizes motifs with symmetric (ACGT) and asymmetric (ACCT) core elements present in both *MAT* promoters. ROM2 antagonizes *trans*-activation of the *DLEC2* promoter by PvALF in transient expression assays. Repression was abolished by mutations that prevented binding of ROM2 to the *DLEC2* seed enhancer region. Moreover, a hybrid protein composed of a PvALF activation domain and the DNA binding and dimerization domain of ROM2 activated gene expression, indicating that ROM2 recognizes the *DLEC2* enhancer in vivo; consequently, ROM2 functions as a DNA binding site-dependent repressor. Supershift analysis of nuclear proteins, using a ROM2-specific antibody, revealed an increase in ROM2 DNA binding activity during seed desiccation. A corresponding increase in *ROM2* mRNA coincided with the period when *DLEC2* mRNA levels declined in embryos. These results demonstrate developmental regulation of the ROM2 repressor and point to a role for this factor in silencing *DLEC2* transcription during late embryogenesis.

INTRODUCTION

Numerous studies have described changes in gene activity and protein accumulation associated with different periods of plant embryogenesis (reviewed in Goldberg et al., 1989, 1994; Galau et al., 1991; Thomas, 1993). At the end of the cotyledon stage, dicot embryos undergo a process of maturation during which they accumulate reserve materials, defense compounds, and desiccation protectants. Based on the pattern of accumulation of numerous mRNAs in cotton embryos, Hughes and Galau (1989) proposed that gene expression after the cotyledon stage comprises two main temporal programs associated with maturation (*MAT*) and postabscission. Included in the *MAT* group were genes encoding vicilin-like storage proteins. Similarly, in French bean, genes *PHSβ* and *DLEC2*, which encode the seed storage protein β-phaseolin (Slightom et al., 1983) and the phytohemagglutinin (PHA) L subunit (Hoffman and Donaldson, 1985), respectively, are highly induced at the onset of maturation (Murray and Kennard, 1984; Staswick and Chrispeels, 1984). Similar patterns have been described for glycinin and conglycinin genes in soybean (Walling et al., 1986; Barker et al., 1988). After a period of intense activity, legume *MAT* gene expression ceases following seed abscission and the onset of desiccation. Although the mechanism of *MAT* gene silencing is not well understood, the process is known to involve transcriptional and post-transcriptional control (Chappel and Chrispeels, 1986; Walling et al., 1986). Further progress in this area requires the identification, cloning, and functional characterization of regulatory factors linking developmental and physiological control of seed maturation and late embryogenesis to specific gene expression.

The hormone abscisic acid (ABA) plays a dual role in seed maturation by inhibiting premature germination of the embryo (vivipary) and inducing *MAT* and late embryogenesis (*LEA*) gene expression (reviewed in Rock and Quatrano, 1994). In Arabidopsis, the ABA-insensitivity loci *ABI3*, *ABI4*, and *ABI5* contribute to normal gene expression during late embryogenesis. Mutations in the *ABI3* locus cause reduced expression of storage protein and *LEA* genes in seed (Koorneef et al., 1989; Finkelstein and Sommerville, 1990; Finkelstein, 1993; Parcy et al., 1994). Mutations in the leafy cotyledon loci *LEC1* and *LEC2* and *FUSCA3* also lead to abnormal phenotypes that may include reduced expression of storage protein and *LEA* genes (Nambara et al., 1992; Bäumlein et al., 1994; Keith et al., 1994; Meinke et al., 1994; West et al., 1994). Of these genetically

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defined loci, only *ABI3* has been cloned (Giraudat et al., 1992), and its protein product has been shown to bear significant homology with Viviparous1, a transcription activator that is required for normal maturation development in maize (McCarty et al., 1991).

Although substantial evidence indicates that *ABI3* regulates gene transcription during seed maturation, further biochemical analyses in this system are hampered by the small size of Arabidopsis seed and embryos. Recently, we reported that PvALF, an ABI3-like protein from the large-seeded legume *Phaseolus vulgaris*, is a transcription activator of *PHSβ* and *DLEC2* genes (Bobb et al., 1995). Although *PvALF* and *ABI3* expression is embryo specific, in both cases it encompasses a longer period of embryogeny than is occupied by *MAT* gene expression (Parcy et al., 1994; Bobb et al., 1995). Therefore, it seems logical to hypothesize that temporal regulation of *MAT* transcription involves additional factors, either coactivators necessary for PvALF function or repressors that antagonize its action at various times of development.

Here, we report the cloning of a cDNA encoding factor, ROM2 (for regulator of *MAT2*). The recombinant ROM2 protein bound specifically to GC-type motifs in *PHSβ* and *DLEC2* promoters. In transient expression assays, ROM2 significantly reduced β-glucuronidase (*GUS*) expression driven by a *DLEC2* promoter and counteracted transcriptional activation by PvALF. *ROM2* mRNA and DNA binding activities in nuclear extracts were upregulated at the same time that phaseolin and PHA mRNAs began to decline. These results indicate that ROM2 contributes to temporal regulation of *DLEC2* gene expression in development.

RESULTS

Cloning of a ROM2 cDNA from Bean by Rapid Amplification of cDNA Ends

The involvement of basic leucine zipper (bZIP) proteins in regulating seed storage protein gene expression has been clearly demonstrated in the case of the maize Opaque2 protein (Lohmer et al., 1991; Schmidt et al., 1992; Yunes et al., 1994). Other bZIP proteins implicated in controlling gene expression in developing embryos are the Opaque2 heterodimerizing proteins OHP1 and OHP2 (Pysh et al., 1993) from maize, EMBP1, which binds to an ABA response element in a wheat *Em* promoter (Guiltinan et al., 1990), and the RITA-1 protein of rice (Izawa et al., 1994). Although there are no corresponding examples from dicots, motifs resembling Opaque2 binding sites are necessary for normal function of the *PHSβ* promoter (Bustos et al., 1991; Kawagoe et al., 1994). Moreover, protein–DNA binding experiments with crude nuclear extracts suggest that immature bean cotyledons contain one or more factors that recognize the *PHSβ* Opaque2-like motifs (M.-S. Chern, H.G. Eiben, and M.M. Bustos, unpublished data).

Initial attempts to screen a bean embryo cDNA library by heterologous hybridization, using a probe corresponding to the Opaque2 bZIP domain, were unsuccessful. Consequently, a more general homology-based strategy was implemented using the method of rapid amplification of cDNA ends (3′ RACE; Frohman et al., 1988). Briefly, cDNA representing poly(A)$^+$ RNA from embryos at 16 to 20 days after flowering (DAF) was synthesized by reverse transcription, using a $(dT)_{17}$ adapter primer (see Methods). The resulting cDNA mixture provided the template for two subsequent rounds of polymerase chain reaction (PCR) amplification, using the 3′ adapter primer and degenerate primers deduced from the sequence of the peptide RK[Q/E/L]SNRESARR, which is conserved in the basic domains of many plant GC-box binding bZIP proteins (Weisshaar et al., 1991). Two primary and four secondary PCRs were performed, using conditions described in Methods. Products of the secondary PCRs were cloned into plasmid pCRII (Invitrogen) and sequenced. Nine of 30 of those clones contained an open reading frame (ORF) coding for part of a basic domain, followed immediately by a leucine zipper. These nine clones represented two different cDNAs, one of which encoded a partial protein with a longer C-terminal tail. In this study, we report additional studies with the longer cDNA.

Sequences located upstream of the basic domain of the cDNA were obtained by the 5′ RACE technique (Frohman et al., 1988), using *Taq* DNA polymerase (Promega) and gene-specific primers deduced from the corresponding 3′ untranslated region. Several positive clones were recovered that cross-hybridized to the 3′ RACE fragment. One of these clones (2-537) was completely sequenced and found to encode a 426–amino acid ORF. To guard against the possibility of PCR-induced mutations that could have occurred during 5′ RACE, new primers were synthesized from the 5′ untranslated region of clone 2-537 and from the untranslated region of 3′ RACE clone 2-327. The ORF was amplified from fresh poly(A)$^+$ RNA, using reverse transcriptase and DNA polymerases—either *Pfu* (Stratagene) or a 40:1 mixture of *Taq* and *Pfu*—resulting in two independent clones, 2-1.4 and 2-1.8, both of which were completely sequenced. Clones 2-537, 2-1.4, and 2-1.8 contain single, nearly identical ORFs. Figure 1 shows the sequence of clone 2-1.8 and the conceptual translation of the ORF encoding ROM2 protein. ROM2 is a 424–amino acid polypeptide with a predicted molecular mass of 45,481 D. Clones 2-1.4 and 2-1.8 contain identical sequences except for a silent, single-base change at nucleotide 691. Clones 2-537 and 2-1.8 differed at positions 691 and 933 and differed by the presence of an extra 6 bp between nucleotides 469 and 470 in clone 2-537 that added two amino acids (EQ) to the potential product. None of the observed changes caused frameshifts or nonsense mutations and are likely to represent different *ROM2* alleles. The ROM2 ORF was preceded by several in-frame stop codons, suggesting that the first amino acid shown in Figure 1 represents the true translation start. At the amino acid level, ROM2 is most closely related (87.5% identity) to a recently published soybean protein, SGBF-2, which was shown by Hong

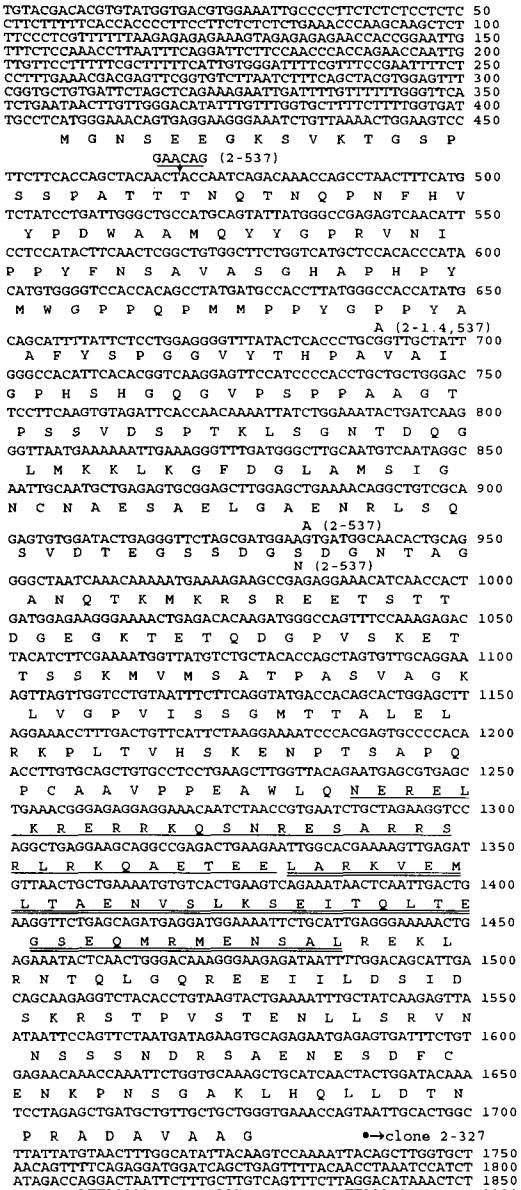


Figure 1. Nucleotide and Deduced Amino Acid Sequences of ROM2.

The complete nucleotide sequences of three cDNA clones containing a full-length ROM2 ORF were determined by the dideoxy method. Shown are the nucleotide sequence and conceptual translation of clone 2-1.8, plus the 3′ untranslated region of 3′ RACE clone 2-327. The two sequences overlap between nucleotides 1280 and 1739. Nucleotides in clones 2-537 and 2-1.4 that differ from clone 2-1.8 are also indicated. A 6-bp insertion at position 460 in clone 2-537 encodes the dipeptide EQ. The basic domain and leucine zipper are highlighted with single and double underlines, respectively. The ROM2 GenBank accession number is U41817.

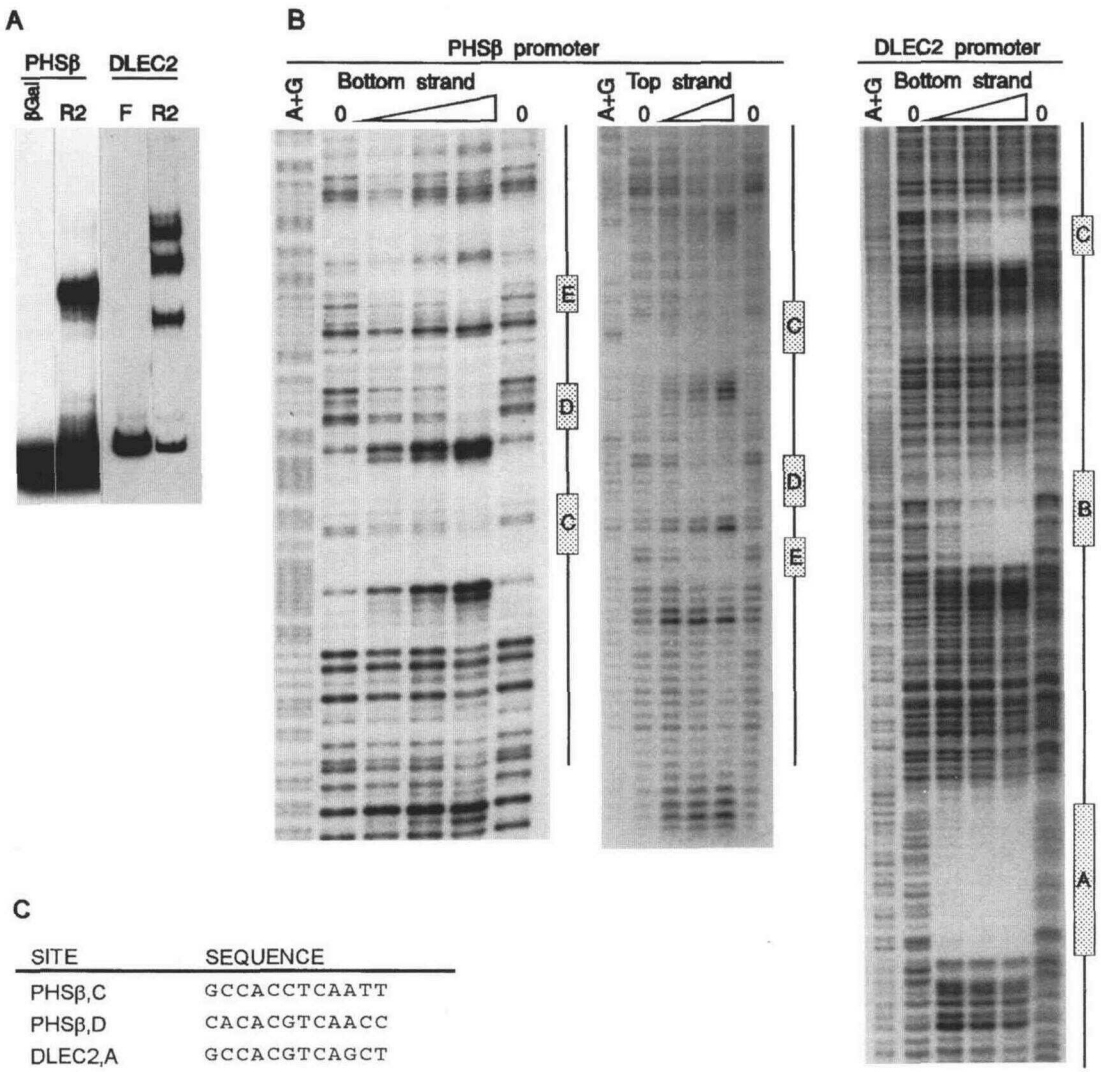
et al. (1995) to interact in vitro with a G-box sequence of an auxin-responsive gene; however, no information has been reported about its function.

Recombinant ROM2 Protein Binds to Multiple Sites in *DLEC2* and *PHS*β Promoters

To investigate the binding of ROM2 polypeptides to *DLEC2* and *PHS*β promoters, a His_6-tagged C-terminal polypeptide (amino acids 268 to 424), comprising the basic domain, leucine zipper, and C-terminal extension of ROM2, was expressed in *Escherichia coli*, using a pET vector (see Methods). A polypeptide of approximately the calculated size was purified by affinity chromatography on a Ni^{2+}-agarose column (Qiagen). Binding of this purified protein to DNA probes prepared from the seed regulatory regions of *DLEC2* (−256 to −5 relative to the transcription initiation site; Riggs et al., 1989) and *PHS*β (UAS1, −302 to −64; Bustos et al., 1991) was studied, using the electrophoretic mobility shift assay (EMSA). As shown in Figure 2A, ROM2 binding to the *DLEC2* promoter probe produced three complexes of different mobilities, indicating the presence of multiple ROM2 recognition sequences. The *PHS*β probe yielded a different pattern consisting of a single major complex. In either case, formation of protein–DNA complexes was dependent on the presence of ROM2, as shown by the absence of binding activity in lanes containing a β-galactosidase (β-Gal) control protein (lane βGal) or no protein (lane F).

DNase I footprinting analysis was used to localize the specific nucleotide motifs recognized by ROM2 on either promoter. As shown in Figure 2B, DNA probes were incubated with increasing amounts of Ni^{2+}-agarose–purified ROM2 protein (ranging from 0 to 400 ng per reaction), cleaved with DNase I, and subsequently analyzed by denaturing gel electrophoresis. ROM2 binding to the *DLEC2* promoter resulted in three distinct areas of protection (sites A, B, and C). Site A overlaps the motif GCCACGTCA centered at position −203, which corresponds to a high-affinity GC box (Izawa et al., 1993). Site B comprises two partially overlapping GC-box motifs, B1 (GCAACGTGTA; bottom strand) and B2 (GCCACCTCA) centered at positions −166 and −159, respectively. Site C also encompasses the motif GCCACCTCA centered at position −99.

Weaker but reproducible DNase I footprints were recorded at five locations (A to E) on the *PHS*β promoter, with the greater degree of protection being apparent at sites C, D, and E. Site C, at position −137, overlaps the motif GCCACCTCA also found in *DLEC2* sites B2 and C. Site D contains the motif CACACGTCA, which is recognized by the recombinant β-Gal–Opaque2 protein (M.-S. Chern, H.G. Eiben, and M.M. Bustos, unpublished data). Site E, at position −94, contains the motif GCGTGTCA, which has only one recognizable high-affinity G-box half-site (Izawa et al., 1993). Weak protection was also recorded at two upstream locations (data not shown): site A (position −249) overlaps the sequence AACACGTGCT, and site B (position −165) overlaps ACCACCTGTA. ROM2 binding also increased cleavage



C

SITE	SEQUENCE
PHSβ,C	GCCACCTCAATT
PHSβ,D	CACACGTCAACC
DLEC2,A	GCCACGTCAGCT
DLEC2,B	GCCACCTCAGCT
DLEC2,C	GCCACCTCAGCT
Consensus	$GCCAC^{G}/_{C}TCA^{G}/_{A}YY$

Figure 2. Electrophoretic Mobility Shifts and DNase I Footprints Caused by Binding of ROM2C to *DLEC2* and *PHSβ* Promoter Sequences.

The recombinant ROM2C and β-Gal proteins were expressed in *E. coli* cells and purified by Ni^{2+} affinity chromatography. The *DLEC2* probe (−256 to −5) was labeled at the 3′ end of the bottom strand. The *PHSβ* bottom strand probe is a 320-bp ClaI-SacI fragment containing phaseolin nucleotides −302 to −64. The *PHSβ* top strand probe is a 311-bp XbaI-XhoI fragment containing the same sequence.

(A) EMSA of ROM2 binding to *DLEC2* and *PHSβ* top strand probes. ROM2C and β-Gal proteins (~35 ng) were incubated with either DNA probe in binding buffer (see Methods), and protein–DNA complexes were separated from free DNA by electrophoresis on a nondenaturing, 4% polyacrylamide gel. βGal, β-Gal protein; F, free DNA probe; R2, ROM2C protein.

(B) DNase I footprinting assays. The DNA probes were incubated with increasing amounts (100 to 400 ng; triangles) of ROM2C protein, cleaved with DNase I, and electrophoresed on a denaturing polyacrylamide gel. Lanes marked 0 denote binding reactions performed without ROM2C protein. A+G, Maxam and Gilbert molecular weight markers. Bars highlight the areas (A to C for *DLEC2*, and A to E for *PHSβ*) protected from DNase I cleavage.

(C) Summary of motifs found within the regions protected from DNase I cleavage by ROM2C.

by DNase I (hypersensitivity) next to all ROM2 binding sites on the phaseolin probe. The sequences of stronger in vitro ROM2 binding sites of *DLEC2* and *PHSβ* promoters are listed in Figure 2C.

The ROM2 binding motifs differ from most plant bZIP binding elements (Izawa et al., 1993) in that they contain a G→C transversion within the central ACGT core. In the phaseolin promoter, site C also forms part of the "vicilin-box," a larger sequence conserved in 7S storage protein (vicilins) promoters of many legumes (Gatehouse et al., 1986). Although indicating that ROM2 binds with similar affinity to sites containing either symmetric (ACGT) or asymmetric (ACCT) core elements, DNase I footprinting experiments did not provide adequate evidence of sequence-specific ROM2 binding to the phaseolin promoter. The specificity of that interaction was addressed in experiments in which wild-type or mutant synthetic sequences were allowed to compete with a radioactively labeled probe for binding to ROM2. As shown in Figure 3A, the probe consisted of a synthetic 46-bp oligonucleotide encompassing *PHSβ* sites C and D. Competitor DNAs contained site A, C, or D either intact (wt) or mutated (mt) by replacing the central 6 bp of each GC box motif. Figure 3B shows that the three wild-type competitors reduced binding of ROM2 to the probe by a similar amount. By contrast, neither of the mutant competitors (Amt, Cmt, and Dmt) affected ROM2–DNA complex formation, demonstrating that ROM2 binds in a sequence-specific manner to multiple sites present on the phaseolin promoter.

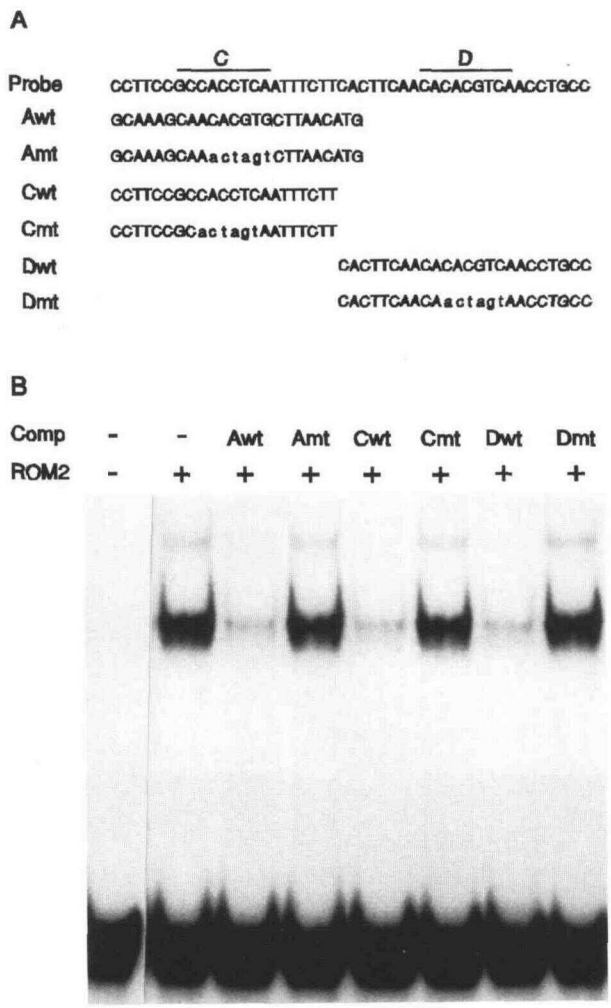


Figure 3. EMSA in the Presence of DNA Competitors.

The ROM2C protein is the same as given in the legend to Figure 2. The probe contains ROM2 binding sites C and D of the *PHSβ* promoter.

(A) Sequence of the double-stranded probe is shown, with sites C (GCCACCTCA) and D (CACACGTCA) indicated. DNA competitors contain sites A, C, or D either intact (Awt, Cwt, or Dwt) or mutated (Amt, Cmt, or Dmt) by substituting the sequence ACTAGT for the central 6 bp of each site.

(B) Electrophoretic mobility shift analysis of protein–DNA complexes produced with ROM2C in the presence of each competitor at ~150-fold molar excess over the probe. "Comp" indicates competitor DNA. (+) and (–) indicate the presence and absence of competitor or ROM2 protein.

Expression of *ROM2* mRNA Is Developmentally Regulated in Bean Embryos

To gain some insight into the probable function of factor ROM2, particularly with regard to the regulation of PHA and phaseolin expression, the time course of *ROM2* mRNA accumulation was analyzed in developing bean embryos. Bean is a predominantly autogamous species in which pollination is completed before opening of the flower corolla. Consequently, the time after maturation of the corolla, expressed here in days after flowering, provides a useful measure of developmental stage. Samples of embryos were collected between 9 and 35 DAF, with the starting and ending points corresponding to early cotyledon and mature (almost dry) stages of embryogenesis. For comparison, samples of leaf, root, and seed pod were also included in the analysis. After extraction of total RNA, the steady state amount of *ROM2* mRNA in each sample was estimated by filter hybridization. As shown in Figure 4, *ROM2* mRNA migrated as a broad band with an average size of ~2.1 kb, consistent with the length of the ROM2 cDNA (2050 bases without the poly(A) tail; Figure 1). The abundance of this mRNA in embryos remained low until 14 DAF and rose thereafter, reaching a maximum at ~27 DAF. A smaller amount of *ROM2* mRNA was also detected in roots, and little was found in leaves and seed pods. The significance of *ROM2* mRNA expression in roots is unclear; screening of a bean genomic DNA library has yielded three *ROM2*-related genes (M.S. Chern and M.M. Bustos, unpublished data), raising the possibility of differential gene expression in embryonic versus vegetative tissues.

The RNA gel blots were also hybridized to cDNA probes for PHA and phaseolin, revealing marked increases in the abundances of both mRNAs between 12 and 14 DAF, which

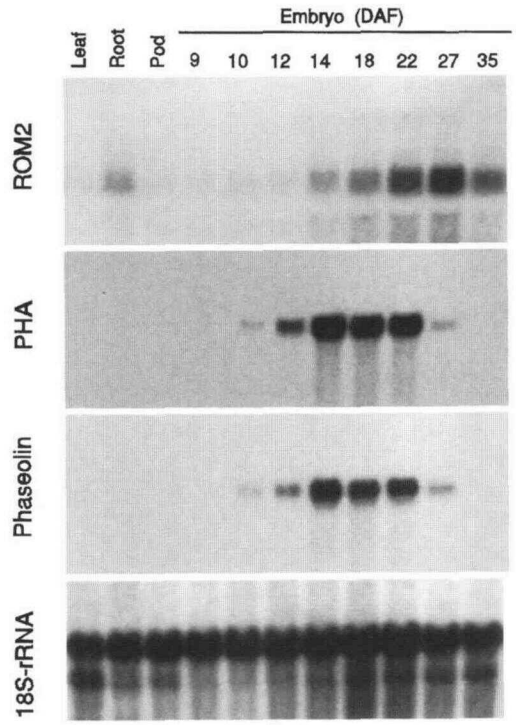


Figure 4. RNA Gel Blot Analysis of *ROM2* Expression.

Total cellular RNA was isolated from leaves, roots, seed pods, and embryos (cotyledons plus embryonic axis) at 9, 10, 12, 14, 18, 22, 27, and 35 DAF. The same amounts (6 μg per lane) of each RNA were separated on a formaldehyde denaturing agarose gel and transferred to nylon membranes. The filters were sequentially hybridized with *ROM2*, *DLEC2* (PHA), phaseolin, and 18S rRNA probes. Exposure times were 3 to 6 hr for phaseolin, PHA, and 18S rRNA and 3 days for *ROM2*.

corresponded with the onset of maturation, as reported earlier (Murray and Kennard, 1984; Staswick and Chrispeels, 1984). The steady state level of either mRNA remained high between 14 and 22 DAF and began to decline thereafter during the period of seed desiccation. Consequently, the most pronounced induction of *ROM2* mRNA corresponded to the time when PHA and phaseolin mRNA levels were down-regulated late in embryogenesis.

An Increase in the Amount of ROM2 DNA Binding Activity in Nuclear Extracts Parallels Changes in Its mRNA

The profile of *ROM2* mRNA provided an indication that this factor might be developmentally regulated. An immunological approach was adopted to study the presence of ROM2 protein in nuclear extracts from bean embryos and to ascertain whether ROM2 DNA binding activity was also developmentally regulated during embryogenesis. Polyclonal antibodies directed against ROM2 were raised in rabbits by immunization with a mixture of two synthetic peptides (MAP200-214 and MAP391-405; see Methods) that were deduced from the conceptual translation of the sequence of ROM2 cDNA clone 2-1.8 and predicted to be of high antigenicity by using the computer program PeptideStructure. Of several antisera tested, αROM2-80 had the highest titer and displayed a high specificity for peptide MAP391-405 (located near the C terminus of ROM2), with no significant reaction to MAP200-214 or any of four other unrelated peptides (data not shown). Antiserum αROM2-80 was also able to immunoprecipitate specifically an in vitro–translated ROM2 protein (H.G. Eiben and M.M. Bustos, unpublished data).

Antiserum αROM2-80 was further tested for its ability to supershift protein–DNA complexes containing recombinant, full-length ROM2 expressed in *E. coli*. Figure 5A shows that the ROM2–DNA complexes (lane ROM2/−Ab) were supershifted when αROM2-80 was added to the binding reaction (lane ROM2/Ab). To demonstrate that the supershifted bands represented ternary complexes of DNA, ROM2, and anti-ROM2 αROM2-80 antibody, controls were performed in which each individual component was either left out or replaced. Consequently, reactions were performed with a DNA probe in which the ACGT core of the ROM2 binding site had been mutated (lane mt), without ROM2 extract (lane Ab), with an extract from *E. coli* expressing β-Gal (lane βGal), and with preimmune serum (lane PI). None of these control reactions produced supershifted bands, confirming the identity of the ternary complexes and the ability of αROM2-80 to cause a ROM2-specific supershift in EMSA. This antiserum was then used to detect ROM2 DNA binding activity in equivalent amounts of nuclear protein extract from bean embryos at 14, 20, and 26 DAF.

Figure 5B shows that a pair of supershifted bands (arrowheads) were formed with the anti-ROM2 αROM2-80 antibody, demonstrating that bean embryos contained one, or perhaps two, nuclear factor(s) antigenically related to cloned ROM2. A GC-box mutant probe (mt) failed to bind nuclear protein under identical conditions, demonstrating the specificity of these interactions. Figure 5B also shows that other sequence-specific binding activities (complexes C1 and C2) were present in the embryos. The abundance of complex C1 remained constant, whereas that of the ROM2 supershifted complexes increased significantly between 14 and 26 DAF. That increase in ROM2 DNA binding activity correlated with the induction of *ROM2* mRNA expression seen during the same period (Figure 4). We conclude that expression of factor ROM2 is developmentally regulated in bean embryos, being higher after the onset of desiccation.

ROM2 Functions as a Repressor That Antagonizes Transcription Activation by PvALF in Bean Cotyledons

Previously, we have demonstrated that factor PvALF functions as a transcription activator of *PHSβ* and *DLEC2* promoters

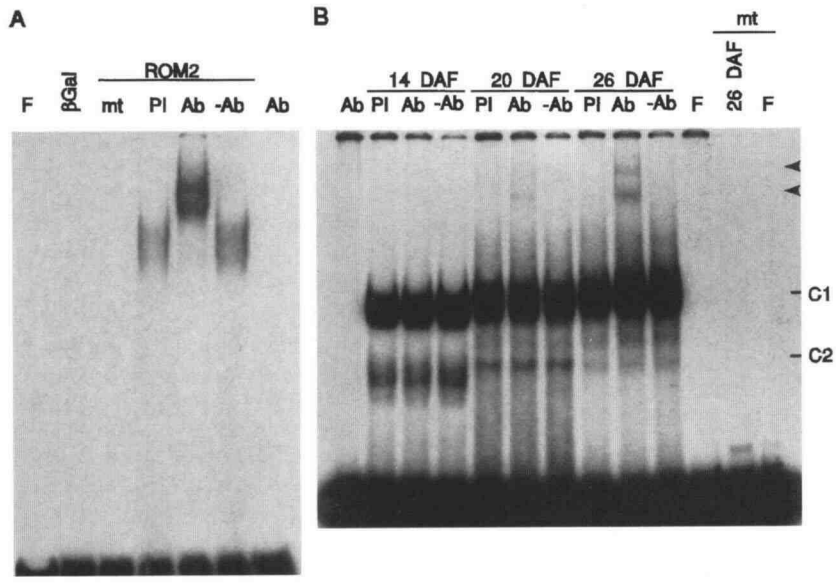


Figure 5. Electrophoretic Mobility Supershift Experiment Using Bean Embryo Nuclear Extracts and Antiserum αROM2-80.

A *DLEC2* probe was incubated with either *E. coli*–expressed protein **(A)** or bean nuclear extract **(B)**. The protein–DNA mixture was further incubated with ammonium sulfate–concentrated sera (anti-ROM2 or preimmune) and then electrophoresed on 4% nondenaturing polyacrylamide gels to resolve protein–DNA complexes from the free DNA probe.

(A) Supershifts of ROM2–DNA complexes with *E. coli*–expressed ROM2 protein. The full-length ROM2 recombinant protein added to reactions (ROM2) was expressed from vector pET15b in BL21(DE3)/pLysS cells. βGal indicates recombinant β-galactosidase protein expressed from pET15b in the same manner. The wild-type probe includes ROM2 site A of the *DLEC2* promoter (−227 to −191); in the mutant probe (mt) the ACGT core of site A has been changed to CTAG.

(B) Supershifts of protein–DNA complexes produced by ROM2 DNA binding activity in bean nuclear extracts. Nuclear extracts were prepared from 14-, 20-, and 26-DAF bean embryos. When present, an amount of nuclear extract equivalent to ~20 mg of fresh tissue was used in each lane. The wild-type probe included *DLEC2* site C (−114 to −89); the mutant probe (mt) had the ACCT core of site C changed to CTAG. Bands that appeared only in the presence of the anti–ROM2-80 antiserum (αROM2-80) are marked by arrowheads. C1 and C2 are other protein–DNA complexes produced by nuclear extracts.

Ab, presence of αROM2-80 antiserum; −Ab, no antiserum; F, free probe; PI, preimmune serum.

(Bobb et al., 1995). The effect of ROM2 on the activity of either promoter was investigated, using DNA bombardment in the presence and absence of exogenous PvALF. Figure 6A depicts the structure of the reporter and effector constructs used in bombardment experiments. As shown in Figures 6B and 6C, in the absence of exogenous PvALF, the effector construct pROM2 reduced *GUS* expression from the *DLEC2* reporter, pPHA, to a greater extent (64%) than from the pPHSβ reporter (33%). The comparatively smaller effect on the latter reporter paralleled the lower affinity of ROM2 for upstream PHSβ sequences (Figure 2). However, the difference could also reflect a higher intrinsic activity of the *DLEC2* reporter construct.

When the PvALF effector (pALF) was incorporated into the assays, both reporters were activated to essentially the same level (pALF–pJIT). However, under these conditions, ROM2 caused the same reduction (~60%) in the activity of the *DLEC2* reporter (pALF–pROM2; Figure 6B) but had a negligible effect on the *PHSβ* promoter (pALF–pROM2; Figure 6C). A *ROM2* antisense construct (pROM2R) had no effect on either reporter, demonstrating that repression required a ROM2 translation product. Furthermore, no repression was observed with a truncated ROM2 protein (encoded by plasmid pROM2C) comprising only the bZIP DNA binding domain and C-terminal tail of ROM2 (similar to the ROM2C protein used in protein–DNA binding experiments in Figures 2 and 3). From the data above, we conclude that repression was likely due to the presence of exogenous ROM2 protein and that DNA binding alone was probably not sufficient for repression. These conclusions were reinforced by the results of experiments described below.

Repression of PvALF-Mediated Activation Depends on the Presence of Intact ROM2 Binding Sites

To determine whether repression required binding of ROM2 protein to its target promoter, the four 5′-ACC/GT-3′ motifs found

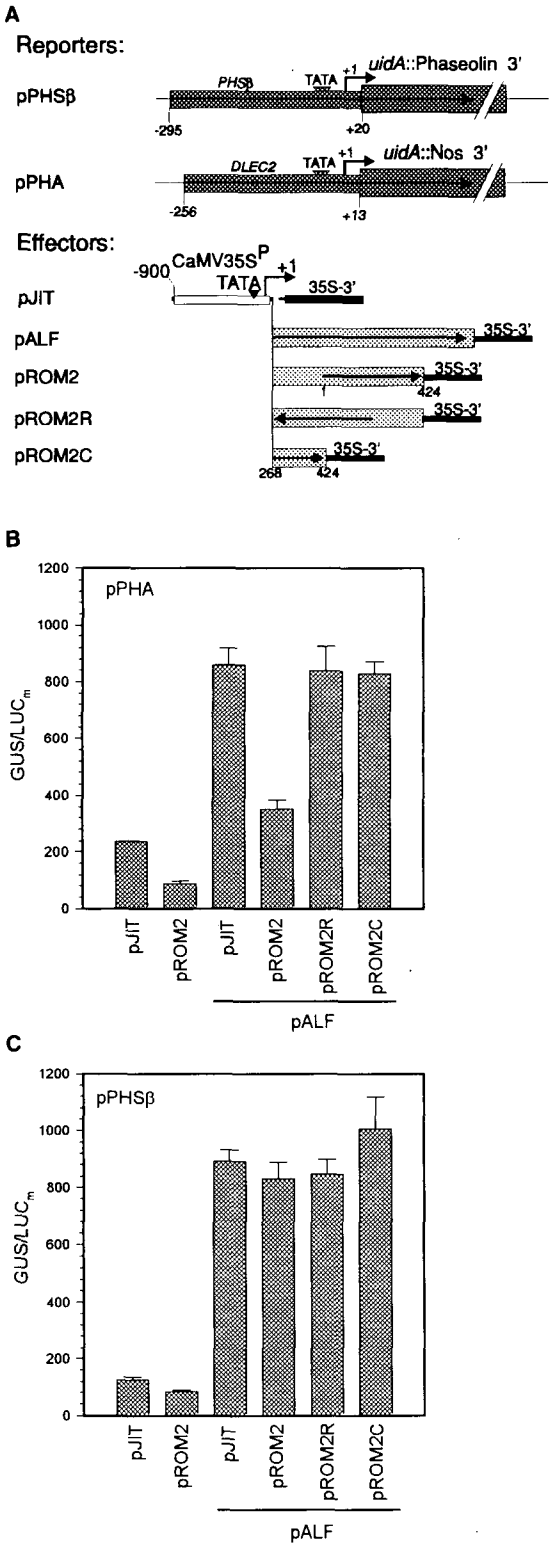


Figure 6. Effect of ROM2 on *GUS* Expression Driven by *DLEC2* and *PHSβ* Promoters.

within *DLEC2* sites A, B, and C localized by DNase I footprinting (Figure 2) were mutated. Wild-type and mutant copies of the enhancer fragment were fused to a minimal cauliflower mosaic virus (CaMV) 35S promoter–*GUS* cassette (p[-64] $35S^P$–GUS), yielding the reporter constructs pPHA35S and pabc35S, whose structures are diagrammed in Figure 7A. Although PvALF is not naturally expressed in leaves, it does enhance transient expression from pPHA35S when transfected in leaf tissues (Bobb et al., 1995). Therefore, leaves offer a potentially better background for testing PvALF–ROM2 interactions with less interference from endogenous PvALF protein and other factors possibly present in cotyledons.

First, the effect of the mutations on PvALF activation was determined in the absence of added ROM2 effector plasmid. Figures 7B (cotyledon) and 7D (leaf) show that the wild-type reporter (pPHA35S) yielded much higher expression in cotyledons than in leaves, either with or without added PvALF. This was consistent with the embryo-specific expression of PHA mRNA (Figure 4) and with previous data showing that expression of the *DLEC2* gene in transgenic tobacco plants is seed specific (Riggs et al., 1989). The wild-type enhancer construct also directed higher *GUS* expression in cotyledons than the mutant (pabc35S), suggesting that one or more of the mutated motifs mediate activation in those organs. By contrast, both reporters yielded the same low level of expression in leaves. A similar amount of *trans*-activation (approximately sixfold) by PvALF was observed from both reporters in cotyledons, whereas in leaves the wild-type reporter responded better (13-fold) than the mutant (sevenfold).

Slices of bean cotyledons (18 to 20 DAF) were transfected by particle bombardment, incubated overnight at 28°C in the dark in agar plates containing hyperosmotic medium, and homogenized in extraction buffer; the extracts were used for GUS and LUC assays. Amounts of DNA per bombardment are as follows: p35S–LUC reference plasmid (0.5 μg), pPHA or pPHSβ reporter plasmids (0.5 μg each), pALF effector (0.25 μg), and pROM2 effector (0.25 μg). Control pJIT plasmid was added as needed to total 2.0 μg of DNA per bombardment. Bars represent the average and standard error of four independent bombardments with each DNA combination.

(A) The structures of reporter and effector constructs. Constructs pPHA and pPHSβ contain the −256 to +13 region of *DLEC2* and the −295 to +20 region of *PHSβ*, respectively. All effector plasmids contain the same CaMV 35S promoter present in pJIT. pALF harbors a complete PvALF ORF; pROM2 contains the 5′ untranslated region and ROM2 ORF of cDNA clone 2-1.8 in the sense orientation; pROM2R has the same DNA fragment as pROM2 but in antisense orientation; pROM2C encodes the bZIP domain and C-terminal tail of ROM2 (ROM2C fragment).

(B) and **(C)** Expression from pPHA and pPHSβ reporters. GUS activities (in picomoles of 4-methylumbelliferone per hour) were normalized to the corresponding value of LUC activity (LUC_m). $LUC_m = 4 \times 10^{-4}$ × relative light units.

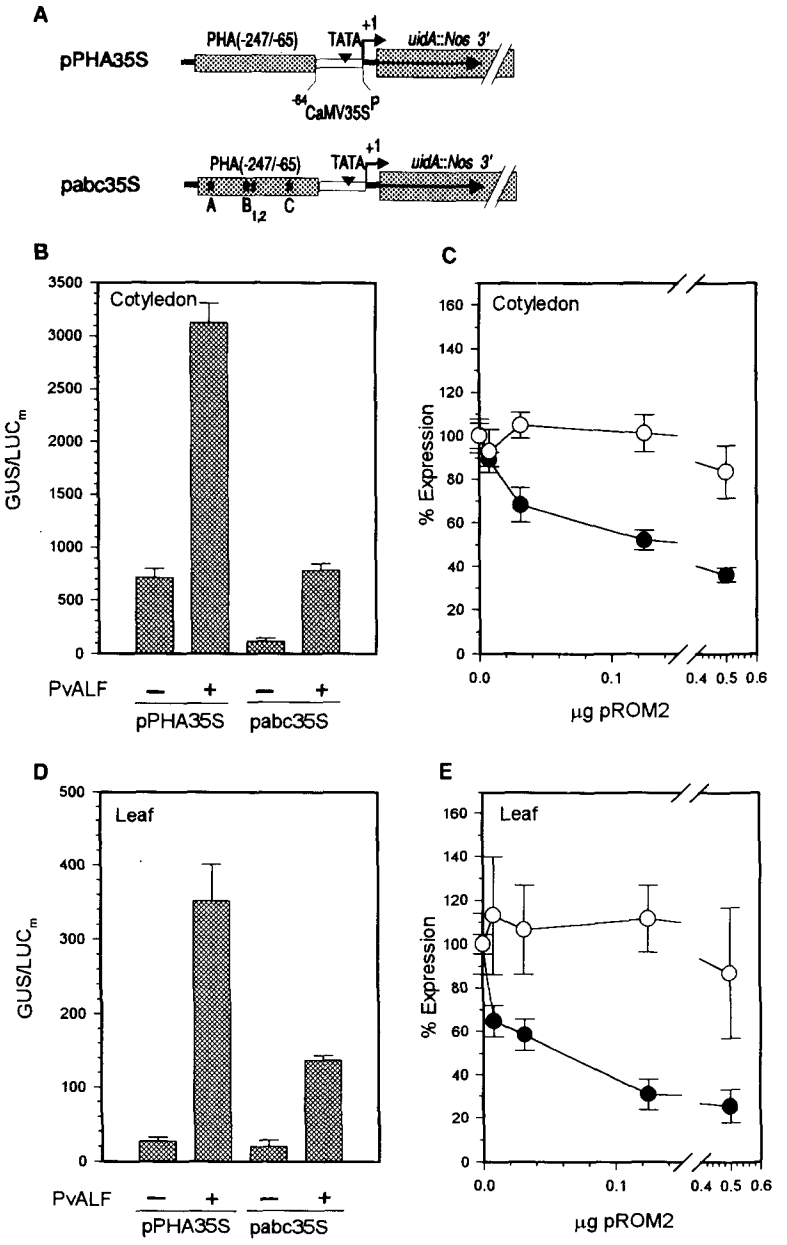


Figure 7. Dependence of Repression on the Amount of ROM2 Effector Plasmid and the Presence of ROM2 Binding Sites in the *DLEC2* Enhancer.

Cotyledon bombardments (**[B]** and **[C]**) were performed as given in the legend to Figure 6; leaf bombardments (**[D]** and **[E]**) were performed on the same day that the leaf discs were prepared, using the method of Bobb et al. (1995). Effectors p35S–LUC, pALF, and pROM2 are as described in Figure 6A. Amounts of DNA per bombardment are as follows: p35S–LUC (0.5 µg), pPHA35S or pabc35S reporter (0.5 µg each), pALF (0.5 µg), pROM2 (0.0078 to 0.5 µg), and enough pJIT to total 2.0 µg of DNA per bombardment. Each data point represents the average and standard error of four independent bombardments.

(A) Structures of reporter constructs. pPHA35S contains the (−247 to −65) enhancer region of gene *DLEC2* joined to a −64 truncated CaMV 35S promoter driving a *uidA* (*GUS*) gene fused to the 3′ end of the nopaline synthase (*Nos*) gene (Jefferson, 1987); pabc35S contains a mutated (−247 to −65) *DLEC2* enhancer. Mutations (indicated by asterisks) changed the ACGT or ACCT motifs in ROM2 binding sites to CTAG.

(B) and **(D)** Control experiments in the absence of added pROM2. (+) and (−) indicate the presence or absence of the PvALF expression vector pALF, respectively. GUS/LUC_m units are described in the legend to Figure 6.

(C) and **(E)** PvALF-activated expression in the presence of increasing amounts (in micrograms) of pROM2. Data are shown as a percentage of expression in the absence of exogenous pROM2. Error bars represent the relative error (Δ) of each percentage value (%N) calculated as Δ = (SE/mean) × %N. Filled circles indicate pPHA35S reporter; open circles indicate pabc35S reporter.

Titration experiments in cotyledons (Figure 7C) and leaves (Figure 7E) were performed by varying the input amount of ROM2 effector plasmid while keeping constant the amounts of pALF effector and wild-type or mutant *DLEC2* reporter DNAs. Data are expressed as a percentage of the initial value measured in the absence of added ROM2. A clear difference in the responses of the two reporter constructs was observed in either organ. Increasing the amount of pROM2 effector caused progressive repression of the wild-type reporter but had no effect on the mutant reporter construct. Significant repression of the wild-type reporter could be seen in both organs even when the amount of ROM2 effector DNA was only a small fraction (1.6%) of the amount of pALF plasmid. These data support the conclusions from the previous section and show that ROM2 repression of PvALF-mediated activation is dependent on the presence of ROM2 binding sites, ruling out the possibility of nonspecific squelching by free ROM2 protein. Moreover, the leaf bombardment data strongly suggested that repression by ROM2 was at least in part due to direct inhibition of PvALF function.

Activation of Expression by a PvALF-ROM2 Fusion Protein Indicates in Vivo Binding of ROM2 to the *DLEC2* Enhancer

The experiments described above established a correlation between in vitro binding of ROM2 to the *DLEC2* enhancer and a requirement of ROM2 target sites for repression of the pPHA35S reporter in vivo. To ascertain whether the ROM2 bZIP domain could recognize the *DLEC2* enhancer in vivo, the strategy of Feldbrügge et al. (1994) was used. Accordingly, a protein fusion was created between the ROM2 C-terminal half (ROM2C, amino acids 268 to 424) and the acidic transcription activation domain of PvALF (ALF-N fragment, amino acids 1 to 243; Bobb et al., 1995) in construct pALF–ROM2 (Figure 8A). Binding of the ROM2 bZIP domain to the *DLEC2* promoter present in pPHA35S would be expected to tether the PvALF activation domain near the site for preinitiation complex formation, resulting in a higher rate of mRNA synthesis (Lin et al., 1991); in turn, that should be detectable as an increase in the amount of *GUS* expression. Two other effector plasmids, pROM2-1.4 and pRT101-O2, were used as controls: pROM2-1.4 lacked the ROM2 5′ untranslated leader present in pROM2; and pRT101-O2 directed expression of the Opaque2 protein, a bZIP transcription activator from maize (Yunes et al., 1994). Figure 8B shows that construct pALF–ROM2 indeed enhanced expression from pPHA35S. Because the untethered PvALF activation domain does not activate transcription in plant cells (Bobb et al., 1995), these results strongly indicated that ROM2 could interact with the *DLEC2* enhancer in vivo. Construct pROM2-1.4 further demonstrated that the ROM2 5′ untranslated sequence was not required for transcription repression. Interestingly, Opaque2 greatly increased expression from the pPHA35S reporter, which was consistent with our previous observation that Opaque2 and ROM2 recognize similar nucleotide

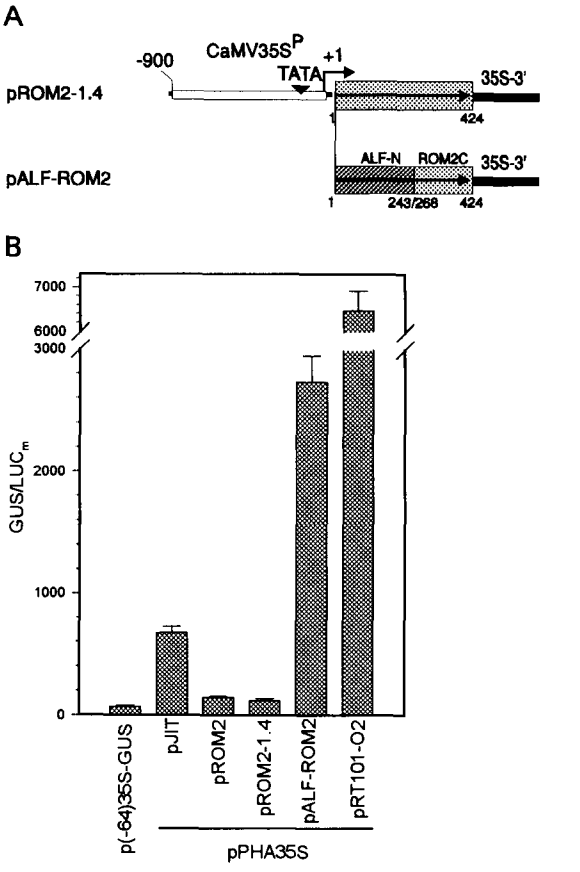


Figure 8. Activation of *GUS* Expression in Vivo by Hybrid Factor ALF–ROM2.

Transfection conditions were as given in the legend to Figure 6. Amounts of DNA per bombardment are as follows: p35S–LUC (0.5 μg); p(-64)-35S–GUS and pPHA35S reporters (0.5 μg each); effector plasmids pALF, pROM2, pROM2-1.4, pALF–ROM2, and pRT101-O2 (0.5 μg each); and enough pJIT to total 2 μg of DNA. Constructs pROM2 and pPHA35S are shown in Figures 6A and 7A. The p(-64)35S–GUS reporter forms the backbone of pPHA35S. pRT101-O2 is an Opaque2 expression plasmid described by Yunes et al. (1994).

(A) Structures of pROM2-1.4 and pALF–ROM2. pROM2-1.4 directs expression of the same ROM2 protein encoded by pROM2 but lacks the 5′ untranslated sequence of cDNA clone 2-1.8. pALF–ROM2 directs expression of the fusion protein ALF–ROM2, which contains the N-terminal activation domain (amino acids 1 to 243) of PvALF and the C-terminal region (amino acids 268 to 424) of ROM2 that includes the bZIP domain.

(B) Expression of activity in the presence of different effectors. Bars represent the average and standard error of four bombardments with each DNA combination. GUS/LUC_m units are described in the legend to Figure 6.

motifs in vitro (M.-S. Chern, H.G. Eiben, and M.M. Bustos, unpublished data).

DISCUSSION

3′ RACE as an Alternative to Conventional cDNA Cloning Strategies

The extreme abundance of phaseolin, PHA, and other maturation-specific mRNAs in bean embryos hinders construction of cDNA libraries representative of low abundance and rare mRNAs, making cloning of transcription factors by conventional procedures very difficult. Here, we successfully employed a PCR-based, 3′ RACE strategy that requires only reverse-transcribed cDNA as the starting material to clone a cDNA encoding factor ROM2. This procedure would be particularly useful for cloning members of poorly conserved gene families that, like plant bZIP transcription factors, share only a short (e.g.,<15), uninterrupted stretch of conserved amino acids. Such conservation is insufficient for conventional PCR amplification schemes requiring two convergent primers, but the difficulty can be overcome by using an oligo(dT) primer that anneals to the poly(A) tail in conjunction with one or more sequence-specific primers. Nested, colinear primers increase specificity and sensitivity, compensating for small amounts of target mRNA and primer degeneracy. Nevertheless, primer design is likely to be the most critical parameter for a successful 3′ RACE protocol; in particular, the number of total permutations in each primer mixture must be kept to a minimum.

Our results suggest that primer populations with a total degeneracy of ~500 permutations are acceptable. More degenerate primers may increase background and lower the yield of specific products, making the task of finding the correct clones too difficult. When isolating new genes by PCR amplification, it is very important to use high-fidelity, thermostable DNA polymerases such as *Pfu* (Stratagene) and to corroborate the sequence and function of the products by analyzing multiple clones.

Function of Factor ROM2

The recombinant ROM2 protein expressed in *E. coli* was shown to bind in vitro, in a sequence-specific manner, to multiple sites on the *DLEC2* and *PHSβ* promoters. Known plant bZIP proteins bind preferentially to motifs containing the core element 5′-ACGT-3′ (Weisshaar et al., 1991), and bases flanking this core determine specificity of DNA recognition (Schindler et al., 1992; Williams et al., 1992). Correspondingly, Izawa et al. (1993) have classified bZIP binding motifs as combinations of G, C, A, and T half-binding sites. Thus, differential affinity of individual bZIP proteins for certain motifs may be an important determinant of how they function in vivo (Foster et al., 1994). Protein–DNA binding experiments demonstrated that ROM2 displays significant affinity for motifs containing the asymmetric core 5′-ACCT-3′ that are poor targets for most other plant bZIP proteins (Izawa et al., 1993; Foster et al., 1994). This feature could have important implications for the function of ROM2 in development by allowing it to interact with promoters containing asymmetric core motifs more readily than with potential competitor proteins.

Several lines of evidence support a role for factor ROM2 in developmental regulation of *DLEC2* gene expression. First, ROM2 binds to a region of the *DLEC2* promoter that is critical for its correct organ and temporal specificity of expression in transgenic tobacco plants (Riggs et al., 1989). Our own data (Figure 7) suggest that at least one of the 5′-ACC/GT-3′ motifs within ROM2 binding sites A, B, and C is required for maximal activity of the *DLEC2* promoter. Second, gene expression of ROM2 is itself developmentally regulated, reaching a maximum during the period of seed desiccation between 25 and 27 DAF. The observed increase in ROM2 DNA binding activity in nuclear extracts occurs well after cell division in the embryo has ceased; therefore, it is more likely to represent an elevated concentration of ROM2 activity per cell than an increase in cell density. Third, the rise in the amounts of *ROM2* mRNA and DNA binding activity coincides with the decline in the steady state level of PHA mRNA. The inverse relationship between the two is consistent with the negative effect that ROM2 has on transcription from the *DLEC2* promoter.

Our data showed that ROM2 had little or no effect on the *PHSβ* promoter in the presence of exogenous PvALF. In view of the relatively low affinity displayed by ROM2 for *PHSβ* upstream sequences (Figure 2), it is possible that repression of PvALF-activated transcription from the *PHSβ* promoter may require a higher concentration of ROM2 protein than can be attained by means of particle bombardment. In footprinting experiments, binding of ROM2 to *PHSβ* upstream sequences also led to increased rates of DNase I cleavage at positions near the sites of protection, which were not seen (or were less obvious) with the *DLEC2* probe. These observations also suggest that ROM2 interacts with the *PHSβ* and *DLEC2* promoters in distinct ways, which could explain their unequal susceptibilities to repression by ROM2. However, because an otherwise subtle difference in the interaction of either promoter with ROM2 could have been magnified by overexpression of PvALF, the significance of the differential effect of ROM2 on these two promoters remains to be determined. It is also important to bear in mind that ROM2 could be one example of a battery of repressors acting in similar ways upon different gene families regulated during embryogenesis.

Of great interest was the negative effect of ROM2 on PvALF-activated transcription, for therein may lie a major clue to its role in the genetic network that regulates maturation-specific gene expression. PvALF is expressed at almost constant levels from the early cotyledon stage to the end of embryogeny (Bobb et al., 1995). A similar pattern was reported previously for the *ABI3* gene in Arabidopsis (Parcy et al., 1994), which has been defined genetically as encoding a component of the ABA signal transduction pathway. However, Parcy et al. (1994)

concluded that changes in the endogenous concentration of ABA could not fully account for the distinct temporal patterns of regulation exhibited by *ABI3*-dependent genes in the *MAT*, *LEA-A*, and *LEA* classes. Therefore, the possibility was raised that other factors contribute to or are responsible for temporal regulation after the cotyledon stage.

In addition to the possible involvement of coactivators, transcription repression provides a straightforward means in which subsets of *ABI3*-dependent genes might be segregated into distinct temporal classes. Accordingly, ROM2 could down-regulate certain *MAT* genes during seed desiccation while allowing continued expression of others. Similarly, repression may also be involved in determining the onset of *MAT* expression and in spatial control within specific regions of the embryo. We have isolated a second bZIP repressor, ROM1, whose expression level is maximal before the induction of phaseolin and PHA mRNA synthesis and which, like ROM2, inhibits transcription activation by PvALF (M.-S. Chern, H.G. Eiben, and M.M. Bustos, unpublished data). Another example of a potential bZIP repressor is the common plant regulatory factor protein of parsley that binds to, and reportedly down-regulates UV-induced activation of, a chalcone synthase promoter (Feldbrügge et al., 1994). Therefore, bZIP transcription repressors may be rather common in plants in which they could participate in a number of diverse phenomena, such as temporal regulation, photomorphogenesis, hormone signaling, and cell- or tissue-specific expression.

Possible Mechanisms of Transcription Repression by ROM2

The mechanism by which factor ROM2 represses *DLEC2* transcription holds the key to its interaction with other components of the maturation regulatory network. The fact that ROM2 had little or no effect on the activities of *PHSβ* and CaMV 35S promoters ruled out nonspecific squelching by free ROM2 protein as a likely mode of action. Moreover, repression of PvALF-activated transcription was dependent on DNA binding and the amount of input *ROM2* effector DNA. DNA binding site–dependent repressors may act in at least three different ways (reviewed in Johnson, 1995). Competitive binding repressors exclude activators from the promoter, a phenomenon that has been described for repressors involved in pattern formation in Drosophila embryos (Small et al., 1991). Repressors may also quench or mask an activator via protein–protein interactions. In this case, each of the interacting partners may be activators, for example, glucocorticoid receptor and AP-1 (Diamond et al., 1990), or one of them may be a "dedicated" repressor protein like the product of the fly Krüppel gene (Licht et al., 1993). Finally, active repressors interfere, directly or via corepressors, with the function of general transcription factors. Recently, examples of direct repression have been reported for the unliganded human thyroid receptor and retinoic acid receptor (Baniahmad et al., 1992; Hörlein et al., 1995) and the *Drosophila even-skipped proteins* (Han and Manley, 1993).

Mutagenesis of ROM2 binding sites on the *DLEC2* enhancer led to a reduction in reporter gene expression in both the presence and absence of PvALF, suggesting that an endogenous factor involved in activation binds to at least some of those sites in vivo. Expression of ROM2 caused a similar reduction in the activity of the intact promoter (Figures 6 to 8), seemingly indicating that ROM2 may act by competing out an endogenous activator. However, the situation is more complicated than that, because analysis of single-site mutants so far indicates that ROM2 may be able to repress transcription from promoter locations that are not important for endogenous activation (M.-S. Chern, A.J. Bobb, H.G. Eiben, and M.M. Bustos, unpublished data). Moreover, deletion of the ROM2 N-terminal half produced a protein that was inactive as a repressor (Figure 6), although it bound to DNA in vitro and, as indicated by the positive effect of the ALF–ROM2 fusion protein (Figure 8), also in vivo. These results suggest that DNA binding alone is not enough for repression, and they imply the existence of a "repression domain" within the N-terminal half of ROM2. Consequently, ROM2 could act by interfering directly with PvALF, a PvALF-associated cofactor or a general transcription factor. Indeed, the interference between ROM2 and PvALF could be mutual.

We have recently determined that PvALF activation requires the presence of intact RY motifs (Hoffman and Donaldson, 1985; Dickinson et al., 1988) that are closely linked to ROM2 binding sites on the *DLEC2* promoter (A.J. Bobb and M.M. Bustos, unpublished data), raising the possibility that RY repeats may somehow contribute to transcription repression by ROM2. In any event, elucidation of the mechanism by which ROM2 represses transcription awaits further study.

METHODS

Plant Materials

Phaseolus vulgaris cv Tendergreen plants were grown in a greenhouse. All materials, including leaves, roots, seed pods, and embryos, were frozen in liquid N_2 immediately after being collected and stored at −70°C until used. For particle bombardment experiments, cotyledon and leaf tissues were collected fresh and used immediately.

Cloning of bZIP Genes by 3′ Rapid Amplification of cDNA Ends

Total RNA was isolated from bean embryos (14 to 15 mm in length) by the guanidinium thiocyanate–cesium chloride method (Sambrook et al., 1989), and poly(A)$^+$ RNA was purified using the Poly-A-Tract mRNA isolation system (Promega, Madison, WI). First-strand cDNA was synthesized from 500 ng of poly(A)$^+$ RNA according to the Superscript Preamplification System (Bethesda Research Laboratories, Gaithersburg, MD); each reverse transcription reaction was primed with a $(dT)_{17}$ adapter (5′-GACTCGAGTCGACATCGATTTTTTTTTTTTT-TTTTT-3′) and incubated at 42°C for 2 hr. After synthesis, the first-strand

cDNA was purified by repeated dilution in 2 mL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and filtration through a Centricon-30 (Amicon, Beverly, MA) three times. The final cDNA pool was stored at −20°C in a volume of ~50 μL.

ROM2 cDNA sequences were amplified using a modification of the 3′ rapid amplification of cDNA ends (RACE) protocol (Frohman et al., 1988). Amplifications were performed by running two nested, 3′-anchored polymerase chain reactions (PCRs), using an adapter primer (5′-GACTCGAGTCGACATCGA-3′) and two sets of degenerate basic leucine zipper (bZIP)–specific primers. The latter were based on the amino acid sequence RK[Q/E/L]SNRESARR found in the basic domains of most plant bZIP proteins. Primer set I, corresponding to the peptide RK[Q/E/L]SNR, comprised oligonucleotide olbZIPd6 (5′-AGRAARSWD-TCHAAYAG-3′, 288 permutations) and oligonucleotide olbZIPd7 (5′-AGRAARSWDTCHAAYCG-3′, 288 permutations); primer set II, corresponding to the peptide RESARR, comprised oligonucleotide olbZIPd3 (5′-MGDGARTCHGCHAGRAG-3′, 216 permutations) and oligonucleotide olbZIPd4 (5′-MGDGARTCHGCHAGRCG-3′, 216 permutations). Nesting of primers from sets I and II resulted in four different PCRs.

The primary 3′ RACE reactions were performed with 3 to 5 μL of first-strand cDNA pool, 2 pmol of adapter primer, and 50 pmol of either primer olbZIPd6 or olbZIPd7 in a 50-μL reaction mixture containing 2.5% formamide, 200 μM deoxynucleotide triphosphates, 1.5 mM $MgCl_2$, and 2.5 units of *Taq* DNA polymerase in the buffer supplied by the manufacturer (Promega). Reactions were first set up without adapter primer and run at 94°C for 4 min, 52°C for 5 min, and 72°C for 20 min to extend the second cDNA strand. After adding adapter primer, amplification was achieved by running 35 cycles (94°C for 2 min, 52°C for 2 min, and 72°C for 2 min) followed by a 10-min extension at 72°C. PCR products were purified on a Centricon-30 filter, and 1 μL of purified product was used for the second reaction, which was performed for 30 cycles, using the same conditions with either primer olbZIPd3 or olbZIPd4. Final PCR products were cloned directly into the pCRII vector (Invitrogen, San Diego, CA), and clones containing inserts larger than 400 bp were partially sequenced. Peptide sequences derived from these clones were compared with known plant bZIP protein sequences to select those that contained a putative bZIP domain.

5′ RACE

5′ RACE was performed using the 5′ RACE system from Bethesda Research Laboratories. Briefly, 500 ng of poly(A)$^+$ RNA was reverse transcribed in a volume of 15 μL (final concentration of 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, and 400 μM each dATP, dCTP, dGTP, and dTTP) at 42°C for 60 min, with 200 units of Superscript II reverse transcriptase and 3 pmol of primer olROM2-1 (5′-ACTCAGATACCATTAGA-3′) deduced from ROM2 3′ untranslated sequence. The product was treated with two units of RNase H at 55°C for 10 min, purified on a Glassmax spin cartridge (Bethesda Research Laboratories), and concentrated by lyophilization. Single-stranded cDNA was redissolved and poly(dC) tailed by incubating with 10 units of terminal deoxynucleotidyl transferase for 10 min at 37°C in a volume of 25 μL (final concentration of 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dCTP). After heat inactivation of terminal deoxynucleotidyl transferase (10 min at 70°C), 5 to 10 μL of poly(dC)-tailed, single-stranded, ROM2-specific cDNA was amplified by PCR with 2.5 units of *Taq* DNA polymerase (Promega), 5′ anchor primer (5′-CUACUACUACUAGGCCACGCGTCGACTAGTACGGGIIG-GGIIGGGIIG-3′), and either ROM2-specific primer olROM2-2 (5′-TTC-ACACCAGTACAGTTAAT-3′) or olROM2-3 (5′-CCCAGTTGAGTATTTCT-CAG-3′), both deduced from the sequence of the ROM2 3′ RACE clone. Reactions were performed in a 50-μL volume containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 400 nM olROM2-2 or olROM2-3, 400 nM anchor primer, and 200 μM each dATP, dCTP, dGTP, and dTTP.

DNA Sequencing

DNA sequencing was done in plasmids, according to the dideoxy sequenase method (U.S. Biochemical, Cleveland, OH). Both strands of the ROM2 clones were sequenced using synthetic oligonucleotide primers.

RNA Gel Blot Hybridization

Total RNA was isolated from seed pods, leaves, and roots of young mature plants and from embryos of different developmental stages, using a hot-phenol–SDS method, as described by Meier et al. (1993). RNA samples were run on a 1.0% formaldehyde denaturing agarose gel (Sambrook et al., 1989), visualized by staining with acridine orange (33 μg/mL in 10 mM sodium phosphate, pH 6.5) to confirm equal loading, transferred to a nylon filter (Nytran; Schleicher & Schuell), and hybridized to random-primed (U.S. Biochemical), ^{32}P-labeled probes by incubating at 43°C for 24 hr with shaking. The hybridization solution contained 50% formamide, 3% SDS, 6 × SSPE (1 × SSPE is 150 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA), 5 × Denhardt's solution (1 × Denhardt's solution is 0.2 mg/mL Ficoll, 0.2 mg/mL polyvinylpyrrolidone, 0.2 mg/mL BSA), and 10% dextran sulfate. The filter was washed at 40°C twice with 2 × SSPE/0.1% SDS, once with 1 × SSPE/0.1% SDS, and once with 0.2 × SSPE/0.2% SDS for 10 min each, covered with Saran wrap while still wet, and exposed to x-ray film. Before rehybridization, the filter was stripped by boiling for 10 min in a solution containing 0.2% SDS and 0.05 × SSPE.

Expression of Recombinant Proteins in *Escherichia coli*

The bZIP domain–containing C-terminal portion of ROM2 (amino acids 268 to 424) was PCR amplified, using primers olROM2-4 (5′-AGTCAT-ATGGCAGCTGTGCCTCCTGAA-3′) and olROM2-5 (5′-TCTGGATCCACA-GCTGATCCATCCTCT-3′), and cloned into the NdeI and BamHI sites of expression vector pET15b (Novagen, Madison, WI), yielding plasmid pET/ROM2-0.6. The junction and bZIP domain regions were sequenced to confirm that they were in the correct open reading frame (ORF). This construct was used to transform *Escherichia coli* host BL21(DE3)/pLysS, harboring the T7 RNA polymerase gene under the control of a *lac* promoter, and transformants were verified by PCR. Cells were pregrown in 100 mL of TB medium (Sambrook et al., 1989) to an OD_{600} of 0.6; expression of recombinant protein was triggered by adding isopropyl-β-D-thiogalactoside to a final concentration of 1 mM, and the cells were incubated for an additional 2 hr at 37°C. Induced bacterial cells were harvested, freeze-thawed three times, and sonicated in sonication buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 0.1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride). Cell debris was spun down, and the supernatant was processed on a Ni^{2+}-NTA-agarose affinity column, according to the manufacturer (Qiagen,

Chatsworth, CA). The recombinant protein (designated ROM2C) was recovered by elution with imidazole buffer, dialyzed into protein buffer S (20 mM Hepes, pH 7.9, 50 mM KCl, 10% glycerol, 0.1 mM Na_2-EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride), and stored at −70°C. For expression of full-length ROM2, the complete ORF of cDNA clone 2-1.4 was PCR amplified using primers olROM2-5 and olROM2-17 (5′-GGGGGATCCCATGGGAAACAGTGAGGAAG-3′) and cloned in frame into the BamHI site of pET15b. Expression of recombinant protein was induced as before. After induction, cells were sonicated directly in protein buffer S, and the extract was incubated at 65°C for 5 min and centrifuged at 14,000 rpm for 5 min at 4°C to remove cell debris. The resulting supernatant was stored at −70°C and used as the source of bacterially expressed full-length ROM2 protein.

Probe Preparation and Electrophoretic Mobility Shift Assay

The *DLEC2* promoter fragment (−256 to −5) was amplified from bean genomic DNA (cv Tendergreen), using primers olPHAL-1 (5′-GGAAGCTTAGTAACATCTGCACTGTGG-3′) and olPHAL-3 (5′-GGTCTAGACCATCATTCTCTTCTCTCTA-3′), purified on a polyacrylamide gel, cloned into the pT7Blue vector (Novagen), and confirmed by sequencing. To prepare the *DLEC2* probe, plasmid DNA was linearized with SpeI, end-filled with the Klenow fragment of DNA polymerase I (U.S. Biochemical) in the presence of α-^{32}P-dCTP, and re-cut with EcoRI to release the probe. The probe was purified on a 5% polyacrylamide gel, extracted with phenol–chloroform (50:50), ethanol precipitated, and resuspended in STE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 50 mM NaCl) to a concentration of ~20,000 cpm/μL. The *PHS*β probes corresponding to the bottom and top strands of fragment UAS1 (*−302* to *−64*) were prepared by a similar procedure.

Synthetic probes *PHS*β C-D (46 bp, positions −143 to −100, gene *PHS*β), DLEC2-A (37 bp, −227 to −191, gene *DLEC2*), and DLEC2-C (26 bp, −114 to −89, gene *DLEC2*) were prepared by annealing two overlapping single-stranded oligonucleotides, followed by Klenow end-filling with α-^{32}P-dCTP in a 20-μL reaction containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 10 pmol annealed oligonucleotides. Resulting probes were purified by electrophoresis on a 12% polyacrylamide gel, eluted into 450 μL of STE buffer, phenol–chloroform extracted, ethanol precipitated, resuspended in STE buffer to 20,000 cpm/μL, and stored at 4°C. Oligonucleotides were as follows: for probe *PHS*β C-D, olVb1-1 (5′-CCTTCCGCCACCTCAATTTCTT-3′) and olVb1-10 (5′-GGCAGGTTGACGTGTGTTGAAGTGAAGAAATTGAGGTGGCGGAAG-3′); for DLEC2-A, olPHA-A1 (5′-GTGAAAGCTGACGTGGCAGCATGCATGGTGAGTGTGA-3′) and prPHA-A2 (5′-TCACACTCACCATGC-3′); for DLEC2-C, olPHAL-Cw1 (5′-GCATGCATGCTGCCACCTCAGCTCCC-3′) and olPHAL-Cw2 (5′-GGGAGCTGAGGTGG-3′).

Protein–DNA binding reactions were incubated either at room temperature for 20 min or on ice for 30 min. The reaction mixture contained 1.5 μL of 0.5 M NaCl, 1.0 μL of 1 μg/μL poly(dI-dC):poly(dI-dC), 1.0 μL (20,000 cpm) of probe, 1.5 μL of 10 × binding buffer (10 × binding buffer contains 100 mM Tris-HCl, pH 7.5, 500 mM NaCl, 25 mM $MgCl_2$, and 5 mM DTT), 5 μL of DNA binding protein in protein buffer S (as given above), and water to a final volume of 15 μL. For competition experiments, cold oligonucleotide competitors were mixed with the probe before adding DNA binding protein. The protein–DNA mixture was run at 140 V on either 4 or 5% nondenaturing polyacrylamide gels in 0.5 × TBE (1 × TBE is 90 mM Tris-borate, 2 mM EDTA, pH 8.0) buffer unless noted otherwise. Gels were dried on Whatman 3MM filter paper and autoradiographed at −70°C with intensifying screens.

DNase I Footprinting Assay

Protein–DNA binding reactions (as before) in a volume of 20 μL were incubated on ice for 20 min and at room temperature for an additional 10 min. A 30-μL volume of DNase I mix (0.6 units of DNase I, 1.67 mM $MgCl_2$, 0.83 mM $CaCl_2$; prewarmed to room temperature) was added to each tube and mixed by pipetting up and down three times. After incubation at room temperature for exactly 60 sec, the reaction was stopped by the addition of 130 μL of DNase I stop solution (384 mM sodium acetate, 64 mM EDTA, 0.28% SDS, 128 μg/mL yeast RNA) supplemented with 100 μg/mL proteinase K. The tubes were then incubated at 37°C for 10 min and extracted once with 180 μL of phenol–chloroform (50:50). The DNA was precipitated from the aqueous phase by adding 360 μL of ethanol, mixing, and incubating for 30 min at room temperature. After spinning (12,000 rpm) for 5 min at room temperature, DNA pellets were rinsed with 70% ethanol, air dried, and resuspended in 3 μL of loading dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF). Resuspended DNA was heated to 80°C for 3 min, chilled immediately on ice, and run on a 6% DNA sequencing gel along with DNA markers from Maxam and Gilbert sequencing reactions. The gels were dried on Whatman 3MM paper and autoradiographed.

Preparation of Nuclear Extracts and Antisera and Electrophoretic Mobility Supershift Assay

Twenty grams of bean embryos from each developmental stage was used to isolate nuclei, from which nuclear extracts were prepared according to the method of Ostergaard-Jensen et al. (1988). Nuclear proteins were precipitated by the addition of ammonium sulfate to 60% saturation, redissolved in 2 mL of protein buffer S, and dialyzed against the same buffer overnight. Antisera against ROM2 were prepared by immunizing New Zealand white rabbits with a mixture of multiple antigenic peptides MAP200-214 (GEGKTETQDGPVSKE) and MAP391-405 (AENESDFCENKPNSG). These peptide sequences were deduced from the conceptual translation of ROM2 cDNA clone 2-1.8 and predicted to be potentially antigenic, using the computer program PeptideStructure (Genetics Computer Group, Madison, WI). Antibodies were precipitated from an aliquot of serum by the addition of ammonium sulfate to 40% saturation, resuspended to its original volume in protein buffer S, supplemented with 0.2 mg/mL of salmon sperm DNA, dialyzed against protein buffer S, and stored at 4°C. Preimmune serum was treated in the identical manner.

Protein–DNA binding reactions were performed as before with 2 μL of *E. coli* extract or nuclear extract in a 10-μL final volume. After incubating on ice for 30 min, 1 μL of ammonium sulfate–fractionated antiserum or preimmune serum was added, and the mixture was incubated further at room temperature for 30 min and on ice for 10 min. The reaction mixtures were analyzed on 4% low ionic strength polyacrylamide gels (0.5 × Tris-glycine buffer is 12.5 mM Tris, 125 mM glycine, pH 8.5) run at 145 V in a cold room. The gels were dried on Whatman 3MM paper or on nylon membranes (to prevent diffusion of the probe) and exposed to x-ray film.

Biolistic Particle Bombardment

Plasmid Construction and Mutagenesis

The luciferase (LUC) reference construct p35S-LUC, driven by the cauliflower mosaic virus (CaMV) 35S promoter, was generated by replac-

ing the β-glucuronidase (*GUS*) coding sequence in vector pBI221 (Clontech, San Francisco, CA) with the *LUC* gene from plasmid pGEM-luc (Promega), using BamHI and SacI sites. The phaseolin reporter construct pPHSβ is the same as phaseolin^{P}–GUS (Bobb et al., 1995). The pPHA reporter plasmid was constructed by amplifying the −256 to +13 (relative to transcription initiation) fragment of gene *DLEC2* from bean (cv Tendergreen) genomic DNA with primers olPHAL-1 and olPHAL-2 (5′-GGTCTAGATGCAATCAAGCATTAACCATC-3′) and substituting the corresponding PCR product for the CaMV 35S promoter of vector pBI221 between the HindIII and XbaI sites. Plasmid p(-64)35S–GUS contains a −64 to +6 fragment of the CaMV 35S promoter cloned between XbaI and NcoI sites of *GUS* vector pBI120 (Jefferson, 1987). To construct pPHA35S, a −247 to −65 *DLEC2* fragment was amplified using primers olPHAL-4 (5′-GGTCTAGAATTCTGCACTGTGGCGCCT-3′) and olPHAL-5 (5′-GGTCTAGAGAAAGACACGGGTG-3′), and the resulting product was inserted between sites for EcoRI and XbaI in p(-64)35S–GUS.

Mutations in the 5′-ACG/CT-3′ cores of *DLEC2* ROM2 binding sites A, B, and C were created by using PCR. First, two partially overlapping DNA fragments were generated using the *DLEC2* enhancer fragment as template, *Pfu* DNA polymerase (Stratagene, La Jolla, CA), and combinations of primers olPHAL-m6 and olPHAL-m11, or olPHAL-m12 and olPHAL-m7. These fragments were gel purified, denatured, reannealed, and extended with *Pfu* in the absence of PCR primers to create full-length mutant template. Then, fresh primers olPHAL-m6 and olPHAL-m7 were added, and the mutant *DLEC2* enhancer fragment was amplified. The final PCR product was subcloned into p(-64)35S–GUS, and its sequence was verified. Oligonucleotides were as follows: olPHAL-m6 (mutation in site A, 5′-GTCTAGAATTCTGCACTGTGGCGCCTTTTTCACACTCACCATGCATGCTGCCctagCAGCTTTCACACTATG-3′); olPHAL-m7 (mutation at site C, 5′-GTCTAGAGAAAGACACGGGTGAAGAGGCGGGAGCTGctagGGCAGCATGCATGCAT-3′); olPHAL-m11 (mutation at site B, 5′-GGCActagGTATGGCCAGTGGTG-3′); and olPHAL-m12 (mutation at site B, 5′-TGGCCATACctagTGCCctagCAGCTTTCTCCCTT-3′).

Effector constructs were prepared by cloning the corresponding genes into the multiple cloning region of pJIT (Bobb et al., 1995) under control of the CaMV 35S promoter. ROM2 sequences were amplified from cDNA clones 2-1.8 and 2-1.4 with primers olROM2-25 (5′-GGGGATCCTGTTAGCACCAAGCTG-3′) and olROM2-24 (5′-GGGGATCCTGTACGACACGTGTATG-3′), or olROM2-25 and olROM2-10 (5′-GGGGATCCGCAAAGATGGGAAACAGTGAGGAAG-3′), respectively. Both fragments were subcloned into pJIT via the BamHI site to generate effector constructs pROM2 (in the sense orientation relative to the 35S promoter), pROM2R (in the antisense orientation), and pROM2-1.4 (in the sense orientation). The latter lacked cDNA sequences upstream of the putative ROM2 translation start codon (Figure 1). Construct pROM2C was created by first cloning a 0.6-kb fragment amplified with primers olROM2-4 and olROM2-5 into vector pT7Blue to introduce a BamHI site upstream of the new ATG start codon contained in primer olROM2-4. The resulting fragment was then subcloned into the BamHI site of pJIT in the sense orientation. To fuse the activation domain of PvALF in frame with the DNA binding domain of ROM2, a PvALF N-terminal fragment (ALF-N) was amplified using primers prALF-HX (5′-GGGAAGCTTCTAGATGGAGTGTGAAGTG-3′) and olAlf-6 (5′-GGGGTCGACCTCTCCTTTGATAACTTGAC-3′), and the C-terminal portion of ROM2 (ROM2C) was amplified with primers olROM2-21 (5′-GGGTCGACGCAGCTGTGCCTCCTGAA-3′) and olROM2-5. Both PCR fragments were cloned separately into the pCRII vector, excised with HindIII and SalI (ALF-N), or SalI and BamHI (ROM2C), and subcloned between the HindIII and BamHI sites of pJIT, generating the effector construct pALF–ROM2.

Preparation of Bean Cotyledons and Leaves

Bean seed pods of 18 to 20 days after fertilization were freshly harvested and surface sterilized by dipping in 95% alcohol for 1 min. Under sterile conditions, thin longitudinal slices were cut from immature cotyledons (12 to 15 mm) and placed in a hyperosmotic liquid medium (Gamborg's B5 salts, 3% sucrose, and 0.7 M mannitol). The slices were laid out on Petri dishes containing the hyperosmotic medium solidified with 0.8% agar, covering a surface ~1 inch in diameter, and incubated at 28°C in the dark for up to 20 hr before bombardment. Leaf samples were freshly obtained from mature leaves of healthy, young plants and surface sterilized with 10% Clorox bleach. Leaf discs ~4 cm in diameter were cut from the leaves, and major veins were avoided. Discs were placed right side up on hyperosmotic nutrient agar plates and used for bombardment the same day.

Particle Bombardment and Measurement of GUS and LUC Activities

Particle bombardments were done with a Bio-Rad (Hercules, CA) PDS-1000/He Biolistic Particle Delivery System. All plasmid DNAs were purified by using the Qiagen midi-prep (or maxi-prep) plasmid preparation kit. Typically, 0.5 μg of LUC reference plasmid, 0.5 μg of *GUS* reporter plasmid, and variable amounts of effector plasmids plus pJIT were used in a total amount of 2 μg of DNA per bombardment. Although four bombardments were usually performed for each set of DNA samples, an amount of DNA sufficient for six identical bombardments was routinely prepared. Tungsten microcarriers (0.3 mg per bombardment; 1.7 μm particle size for cotyledons and 1.3 μm for leaves) were washed three times with 1 mL of ethanol and twice with 1 mL of H_2O, resuspended by vortexing in H_2O to a concentration of 1.8 mg/50 μL, and dispensed into 50-μL aliquots. To each aliquot was added 12 μg of DNA (in a 20-μL volume), 50 μL of 2.5 M $CaCl_2$, and 20 μL of 0.1 M spermidine. The DNA-coated microcarriers were spun down, rinsed with 250 μL of ethanol, and resuspended in 65 μL of ethanol. The particles were dispersed by brief sonication and immediately spotted onto macrocarriers in 10-μL aliquots. After drying, the microcarriers were bombarded onto bean tissues at 1550 psi.

Tissues were incubated overnight at 28°C in the dark, and ground with a mortar and pestle in 600 μL of GUS/LUC buffer (Montgomery et al., 1993) containing 100 mM potassium phosphate, pH 8.0, 1 mM EDTA, 10 mM DTT, and 5% glycerol. Cell debris was sedimented by centrifugation at 14,000 rpm for 10 min in a cold room. GUS activity was measured as described by Jefferson (1987), using a fluorometer (TKO100; Hoeffer, San Francisco, CA), usually by incubating a mixture of 50 μL of protein extract and 50 μL of 4 mM 4-methylumbelliferyl-β-D-glucuronide at 37°C for 1 to 2 hr.

Measurement of LUC activity was performed on a luminometer (No. LB9501; Berthold, Germany), using the LUC assay reagent (Promega) containing 20 mM tricine, pH 7.8, 1.07 mM $(MgCO_3)_4Mg(OH)_2{\cdot}5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 μM coA, 470 μM luciferin, and 530 μM ATP. LUC activity assays were done according to the manufacturer by measuring luminescence for 10 sec after mixing 20 μL of protein extract with 100 μL of LUC assay reagent added into the cuvette using an automated injection device. The raw activity (in light units) from 20 μL of extract was multiplied by a factor of 10^{-5}, and the corrected value (LUC_m) was divided into the GUS activity (in picomoles of 4-methylumbelliferone per hour), yielding the GUS/LUC_m ratio of each sample.

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REFERENCES

Baniahmad, A., Kohne, A.C., and Renkawitz, R. (1992). A transferable silencing domain is present in the thyroid hormone receptor, in the v-erbA oncogene product and in the retinoic acid receptor. EMBO J. **11,** 1015–1023.

Barker, S.J., Harada, J.J., and Goldberg, R.B. (1988). Cellular localization of soybean storage protein mRNA in transformed tobacco plants. Proc. Natl. Acad. Sci. USA **85,** 458–462.

Baümlein, H., Miséra, S., Luerssen, H., Kölle, K., Horstmann, C., Wobus, U., and Müller, A.J. (1994). The *FUS3* gene of *Arabidopsis thaliana* is a regulator of gene expression during late embryogenesis. Plant J. **6,** 379–387.

Bobb, A.J., Eiben, H.G., and Bustos, M.M. (1995). PvAlf, an embryo-specific acidic transcriptional activator enhances gene expression from phaseolin and phytohemagglutinin promoters. Plant J. **8,** 331–343.

Bustos, M.M., Begum, D., Kalkan, F.A., Battraw, M.J., and Hall, T.C. (1991). Positive and negative *cis*-acting DNA domains are required for spatial and temporal regulation of gene expression by a seed storage protein promoter. EMBO J. **10,** 1469–1479.

Chappell, J., and Chrispeels, M.J. (1986). Transcriptional and post-transcriptional control of phaseolin and phytohemagglutinin gene expression in developing cotyledons of *Phaseolus vulgaris.* Plant Physiol. **81,** 50–54.

Diamond, M.I., Miner, J.N., Yoshinaga, S.K., and Yamamoto, K.R. (1990). Transcription factor interactions: Selectors of positive or negative regulation from a single DNA element. Science **249,** 1266–1272.

Dickinson, C.D., Evans, R.P., and Nielsen, N.C. (1988). RY repeats are conserved in the 5′-flanking region of legume seed protein genes. Nucleic Acids Res. **16,** 371.

Feldbrügge, M., Sprenger, M., Dinkelbach, M., Yazaki, K., Harter, K., and Weisshaar, B. (1994). Functional analysis of a light-responsive plant bZIP transcriptional regulator. Plant Cell **6,** 1607–1621.

Finkelstein, R.R. (1993). Abscissic acid–insensitive mutations provide evidence for stage-specific signal pathways regulating expression of an Arabidopsis late embryogenesis-abundant (*lea*) gene. Mol. Gen. Genet. **238,** 401–408.

Finkelstein, R.R., and Sommerville, C. (1990). Three classes of abscisic acid (ABA)-insensitive mutations of Arabidopsis define genes that control overlapping subsets of ABA responses. Plant Physiol. **94,** 1172–1179.

Foster, R., Izawa, T., and Chua, N.-H. (1994). Plant bZIP proteins gather at ACGT elements. FASEB J. **8,** 192–200.

Frohman, M.A., Dush, M.K., and Martin, G.R. (1988). Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA **85,** 8998–9002.

Galau, G.A., Jakobsen, K.S., and Hughes, D.W. (1991). The controls of late dicot embryogenesis and early germination. Physiol. Plant. **81,** 280–288.

Gatehouse, J.A., Evans, I.M., Croy, R.R.D., and Boulter, D. (1986). Differential expression of genes during legume seed development. Philos. Trans. R. Soc. Lond. B **314,** 367–384.

Giraudat, J., Hauge, B.M., Valon, C., Smalle, J., and Parcy, F. (1992). Isolation of the Arabidopsis *ABI3* gene by positional cloning. Plant Cell **4,** 1251–1261.

Goldberg, R.B., Barker, S.J., and Perez-Grau, L. (1989). Regulation of gene expression during plant embryogenesis. Cell **56,** 149–160.

Goldberg, R.B., de Paiva, G., and Yadegari, R. (1994). Plant embryogenesis: Zygote to seed. Science **266,** 605–614.

Guiltinan, M.J., Marcotte, W.R., and Quatrano, R.S. (1990). A plant leucine zipper protein that recognizes an abscisic acid response element. Science **250,** 267–271.

Han, K., and Manley, J.L. (1993). Transcriptional repression by the *Drosophila* even-skipped protein: Definition of a minimal repression domain. Genes Dev. **7,** 491–503.

Hoffman, L.M., and Donaldson, D.D. (1985). Characterization of two *Phaseolus vulgaris* phytohemagglutinin genes closely linked on the chromosome. EMBO J. **4,** 883–889.

Hong, J.C., Cheong, Y.H., Nagao, R.T., Bahk, J.D., Key, J.L., and Cho, M.J. (1995). Isolation of two soybean G-box binding factors which interact with a G-box sequence of an auxin-responsive gene. Plant J. **8,** 199–211.

Hörlein, A.J., Näär, A.M., Heinzel, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamei, Y., Söderström, M., Glass, C.K., and Rosenfeld, M.G. (1995). Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. Nature **377,** 397–404.

Hughes, D.W., and Galau, G.A. (1989). Temporally modular gene expression during cotyledon development. Genes Dev. **3,** 358–369.

Izawa, T., Foster, R., and Chua, N.-H. (1993). Plant bZIP protein DNA binding specificity. J. Mol. Biol. **230,** 1131–1144.

Izawa, T., Foster, R., Nakajima, M., Shimamoto, K., and Chua, N.-H. (1994). The rice bZIP transcriptional activator RITA-1 is highly expressed during seed development. Plant Cell **6,** 1277–1287.

Jefferson, R.A. (1987). Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol. Biol. Rep. **5,** 387–405.

Johnson, A.D. (1995). The price of repression. Cell **81,** 655–658.

Kawagoe, Y., Campell, B.R., and Murai, N. (1994). Synergism between CACGTG (G-box) and CACCTG *cis*-elements is required for activation of the bean seed storage protein β-phaseolin gene. Plant J. **5,** 885–890.

Keith, K., Kraml, M., Dengler, N.G., and McCourt, P. (1994). *fusca3*: A heterochronic mutation affecting late embryo development in Arabidopsis. Plant Cell **6**, 589–600.

Koorneef, M., Hanhart, C.J., Hilhorst, H.W.M., and Karssen, C.M. (1989). In vivo inhibition of seed development and reserve protein accumulation in recombinants of abscisic acid biosynthesis and responsiveness mutants in *Arabidopsis thaliana*. Plant Physiol. **90**, 463–469.

Licht, J.D., Ro, M., English, M.A., Grossel, M., and Hansen, U. (1993). Selective repression of transcriptional activators at a distance by the *Drosophila* Krüppel protein. Proc. Natl. Acad. Sci. USA **90**, 11361–11365.

Lin, Y.-S., and Green, M.R. (1991). Mechanism of action of an acidic transcriptional activator in vitro. Cell **64**, 971–981.

Lohmer, S., Maddaloni, M., Motto, M., Di Fonzo, N., Hartings, H., Salamini, F., and Thompson, R.D. (1991). The maize regulatory locus *Opaque2* encodes a DNA binding protein which activates the transcription of the *b-32* gene. EMBO J. **10**, 617–624.

McCarty, D.R., Hattori, T., Carson, C.B., Vasil, V., Lazar, M., and Vasil, I.K. (1991). The *Viviparous*-1 developmental gene of maize encodes a novel transcriptional activator. Cell **66**, 895–905.

Meier, B.M., Shaw, N., and Slusarenko, A.J. (1993). Spatial and temporal accumulation of defense gene transcripts in bean (*Phaseolus vulgaris*) leaves in relation to bacteria-induced hypersensitive cell death. Mol. Plant-Microbe Interact. **6**, 453–466.

Meinke, D.W., Franzmann, L.H., Nickle, T.C., and Yeung, E.C. (1994). *Leafy Cotyledon* mutants of Arabidopsis. Plant Cell **6**, 1049–1064.

Montgomery, J., Goldman, S., Deikman, J., Margossian, L., and Fischer, R.L. (1993). Identification of an ethylene-responsive region in the promoter of a fruit ripening gene. Proc. Natl. Acad. Sci. USA **90**, 5939–5943.

Murray, M.G., and Kennard, W.C. (1984). Altered chromatin conformation of the higher plant gene phaseolin. Biochemistry **23**, 4225–4232.

Nambara, E., Naito, S., and McCourt, P. (1992). A mutant of Arabidopsis which is defective in seed development and storage protein accumulation is a new *abi3* allele. Plant J. **2**, 435–441.

Ostergaard-Jensen, E., Marcker, K.A., Schell, J., and de Bruijn, F.J. (1988). Interaction of a nodule specific, *trans*-acting factor with distinct DNA elements in the soybean leghaemoglobin *lbc3* 5′ upstream region. EMBO J. **7**, 1265–1271.

Parcy, F., Valon, C., Raynal, M., Gaubier-Comella, P., Delseny, M., and Giraudat, J. (1994). Regulation of gene expression programs during Arabidopsis seed development: Roles of the *ABI3* locus and of endogenous abscisic acid. Plant Cell **6**, 1567–1582.

Pysh, L.D., Aukerman, M.J., and Schmidt, R.J. (1993). OHP1: A maize basic domain/leucine zipper protein that interacts with Opaque2. Plant Cell **5**, 227–236.

Riggs, C.D., Voelker, T.A., and Chrispeels, M.J. (1989). Cotyledon nuclear proteins bind to DNA fragments harboring regulatory elements of phytohemagglutinin genes. Plant Cell **1**, 609–621.

Rock, C.D., and Quatrano, R.S. (1994). Insensitivity is in the genes. Curr. Biol. **4**, 1013–1015.

Sambrook, J., Frisch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Schindler, U., Terzaghi, W., Beckmann, H., Kadesch, T., and Cashmore, A.R. (1992). DNA binding site preferences and transcriptional activation properties of the Arabidopsis GBF bZIP proteins. EMBO J. **11**, 1274–1289.

Schmidt, R.J., Ketudat, M., Aukerman, M.J., and Hoschek, G. (1992). Opaque2 is a transcriptional activator that recognizes a specific target site in the 22-kD zein genes. Plant Cell **4**, 689–700.

Slightom, J.L., Sun, S.M., and Hall, T.C. (1983). Complete nucleotide sequence of a French bean storage protein gene: Phaseolin. Proc. Natl. Acad. Sci. USA **82**, 3320–3324.

Small, S., Kraut, R., Hoey, T., Warrior, R., and Levine, M. (1991). Transcriptional regulation of a pair-rule stripe in *Drosophila*. Genes Dev. **5**, 827–839.

Staswick, P., and Chrispeels, M.J. (1984). Expression of lectin genes during seed development in normal and phytohemagglutinin deficient cultivars of *Phaseolus vulgaris*. J. Mol. Appl. Genet. **2**, 525–535.

Thomas, T.T. (1993). Gene expression during plant embryogenesis and germination: An overview. Plant Cell **5**, 1401–1410.

Walling, L., Drews, G.N., and Goldberg, R.B. (1986). Transcriptional and post-transcriptional regulation of soybean seed protein mRNA levels. Proc. Natl. Acad. Sci. USA **83**, 2123–2127.

Weisshaar, B., Armstrong, G.A., Block, A., da Costa e Silva, O., and Hahlbrock, K. (1991). Light-inducible and constitutively expressed DNA binding proteins recognizing a plant promoter element with functional relevance in light responsiveness. EMBO J. **10**, 1777–1786.

West, M.A.L., Yee, K.M., Danao, J., Zimmerman, J.L., Fischer, R.L., Goldberg, R.B., and Harada, J.J. (1994). *LEAFY COTYLEDON1* is an essential regulator of late embryogenesis and cotyledon identity in Arabidopsis. Plant Cell **6**, 1731–1745.

Williams, M.E., Foster, R., and Chua, N.-H. (1992). Sequences flanking the hexameric G-box core CACGTG affect the specificity of protein binding. Plant Cell **4**, 485–496.

Yunes, J.A., Neto, G.C., da Silva, M.J., Leite, A., Ottoboni, L.M.M., and Arruda, P. (1994). The transcriptional activator Opaque2 recognizes two different target sequences in the 22-kD-like α-prolamin genes. Plant Cell **6**, 237–249.