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The *BELL1* Gene Encodes a Homeodomain Protein Involved in Pattern Formation in the Arabidopsis Ovule Primordium

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Summary

Ovule development in Arabidopsis involves the formation of three morphologically defined proximal–distal pattern elements. Integuments arise from the central pattern element. Analysis of Bell1 (Bel1) mutant ovules indicated that *BEL1* was required for integument development. Cloning of the *BEL1* locus reveals that it encodes a homeodomain transcription factor. Prior to integument initiation, *BEL1* RNA localizes to the central domain, providing molecular evidence for a central pattern element. Therefore, proximal–distal patterning of the ovule involves the regulated expression of the *BEL1* gene that controls integument morphogenesis. A model for *BEL1* function is evaluated with regard to new data showing the expression pattern of the floral homeotic gene *AGAMOUS* (*AG*) early in wild-type and Bel1 ovule development.

Introduction

Pattern formation is the process by which positional information is imparted and then perceived by competent tissues (Slack, 1983). Morphogenesis is a consequence of the elaboration of positional information. In plants, the mechanisms that regulate pattern formation and morphogenesis are just beginning to be defined. For example, genetic and molecular studies of flower development suggest that the sequential activation of and interactions between members of the MADS box family of transcription factors regulate patterning of the floral whorls (Coen and Meyerowitz, 1991). Similarly, apical–basal axis pattern elements of the embryo may be defined through genetic analysis of mutants (Barton and Poethig, 1993; Jürgens, 1995) and marked by regional specific expression of seed storage proteins (Goldberg et al., 1994).

Ovule development provides a simple system to study pattern formation and morphogenesis. Morphological analysis of ovule development in Arabidopsis suggests that ovule primordia consists of three discrete primary pattern elements that are the nucellar, chalazal (or central), and funicular domains (Schneitz et al., 1995). Elaboration of these pattern elements results in the morphogenesis of the female gametophyte from the nucellar domain and the integuments from the central domain. The chalaza is defined as the region of the ovule where the integuments merge with the funiculus (Esau, 1960). Ovule development in the model system Arabidopsis has been described extensively (Mansfield et al., 1991; Modrusan et al., 1994; Robinson-Beers et al., 1992; Schneitz et al., 1995); however, the molecular mechanisms that generate positional information within the ovule, and how these signals are interpreted, are unknown.

Recessive mutations in the *BELL1* (*BEL1*) gene primarily affect integument morphogenesis and identity (Modrusan et al., 1994; Robinson-Beers et al., 1992). At the stage when both integuments are formed in wild type, Bel1 mutant ovules do not form an inner integument. Instead, only a single abnormal structure is formed in the position of the outer integument. Later in development, some of these abnormal integument-like structures may become homeotically converted to carpel-like structures (Modrusan et al., 1994; Ray et al., 1994). In addition, female gametophyte development is arrested, and Bel1 plants are female sterile.

The formation of carpel-like structures from the integument-like structures has been correlated with expression of *AGAMOUS* (*AG*) late in Bel1 ovule development (Modrusan et al., 1994; Ray et al., 1994). *AG* is a member of the MADS box family of transcription factors that functions in the specification of stamen and carpel organ identity and in determination of the floral meristem (Coen and Meyerowitz, 1991). Transgenic tobacco plants that express a Brassica homolog of *AG* (*BAG*) gene, in addition to converting outer whorls of the flower to carpels, show a homeotic conversion of the ovule primordia to carpel-like structures (Mandel et al., 1992). When expressed in Arabidopsis, the same construct was reported to phenocopy Bel1 in all respects (Ray et al., 1994). It has been proposed that *BEL1* negatively regulates *AG* in the ovule (Modrusan et al., 1994; Ray et al., 1994).

In this paper, we describe the cloning and expression pattern of the *BEL1* gene. Within the ovule, in situ localization of the *BEL1* RNA demonstrates at the molecular level the presence of a chalazal domain and suggests that pattern formation within the ovule involves the regulated expression of the *BEL1* gene. The predicted BEL1 protein encodes a homeodomain DNA-binding motif, indicating that *BEL1* controls integument morphogenesis and identity through the regulation of downstream genes. We show that the expression patterns of *BEL1* and *AG* overlap in the ovule, suggesting that *BEL1* does not act as a negative transcriptional regulator of *AG* in the integuments.

Results

Molecular Cloning of *BEL1*

Earlier, we described the isolation of the *bel1-2* and *bel1-3* alleles from T-DNA mutagenized populations and showed that they were derived from independent T-DNA lines (Mo-

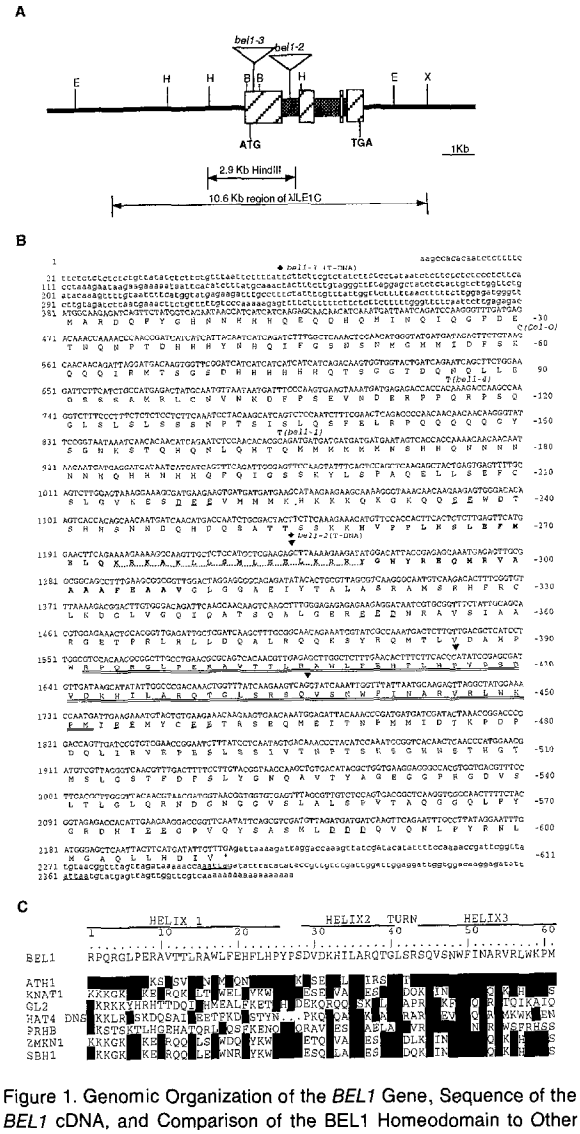

Figure 1. Genomic Organization of the *BEL1* Gene, Sequence of the *BEL1* cDNA, and Comparison of the BEL1 Homeodomain to Other Plant Homeodomains

(A) Restriction map of the *BEL1* genomic region. Restriction sites are indicated as follows: HindIII (H), XbaI (X), EcoRI (E), and BglII (B). Exons are delimited by hatched boxes and introns by closed boxes. The genomic regions used to screen a cDNA library (2.9 kb) and to complement the *bel1-2* allele are indicated below.

(B) The cDNA sequence shown was obtained from the sequences of a 2.1 kb flower and 2.3 kb leaf cDNA clone (see Experimental Procedures). Significant features of the nucleotide and amino acid sequence are indicated as follows: closed triangles indicate the positions of the introns; closed diamonds indicate the positions of the T-DNA insertions; the homeodomain is doubly underlined; the putative amphipathic helix is in bold; the candidate nuclear localization sequence is marked by a dotted underline; acidic regions are indicated by the singly underlined amino acids; potential polyadenylation signals are indicated by a single underline. Differences in sequence between Landsberg erecta and Columbia ecotypes and the mutations in *bel1-1* and *bel1-4* are shown above the nucleotide sequence.

(C) The BEL1 homeodomain was aligned to homeodomains representing most major classes of plant homeobox genes. Percent identities given below are only for the homeodomain. For each class, an Arabidopsis protein was used for comparison, except for the KN1-like family, where maize (KN1, 44% identity; Vollbrecht et al., 1991) and soybean (SBH1, 44% identity; Ma et al., 1994) proteins were also included. The classes were those defined by Kerstetter et al. (1994), except for inclusion of ATH (72% identity; Quaedvlieg et al., 1995) and are as follows: class 1 KN1-like; KNAT1 (44% identity; Lincoln et al., 1994); HD-ZIP; HAT4 (26% identity; Ruberti et al., 1991; Schena and Davis, 1992); PHD-finger; PRHB (24% identity; Korfhage et al., 1994); GL2 (24% identity; Rerie et al., 1994). The homeodomains from each peptide were compiled such that the conserved amino acids aligned. Black blocks indicate identity to BEL1 sequence.

drusan et al., 1994). To isolate the *BEL1* gene, plant sequences flanking the T-DNA insertion in *bel1-2* were identified and used to clone the corresponding wild-type genomic region (see Experimental Procedures; Figure 1A) and transcript (Figure 1B). The longest cDNA clone obtained was 2330 nt. Positions of introns (Figure 1A) were determined by comparing the genomic and cDNA sequences.

To verify that we had isolated the *BEL1* gene, we complemented the Bel1 phenotype by introducing 10.6 kb of the wild-type genomic locus (Figure 1A) into a homozygous *bel1-2* background (see Experimental Procedures). Homozygous *bel1-2* plants containing the wild-type transgene showed a normal ovule morphology and were fertile, indicating that the transgene had complemented the Bel1 phenotype (data not shown).

The *BEL1* Gene Encodes a Homeodomain Protein

To understand the function of the *BEL1* gene, we analyzed the transcript defined by the cDNA clones. As shown in Figure 1B, the *BEL1* cDNA sequence contains an open reading frame that encodes a protein of 611 amino acids. The predicted BEL1 protein contains a homeodomain DNA-binding motif that extends from residues 392–452 (Figure 1C). When compared with representatives of the major classes of plant homeodomains (Kerstetter et al., 1994), BEL1 is similar to the homeodomain of class 1 *KN1*-like genes, but lacks the ELK domain that distinguishes this class (Kerstetter et al., 1994; Vollbrecht et al., 1993). Overall, BEL1 is most similar to ARABIDOPSIS THALIANA HOMEOBOX1 (ATH1) (Figure 1C) (Quaedvlieg et al., 1995). Therefore, BEL1 and ATH1 are members of a novel family of plant homeodomain proteins. Additional members of this family have been identified by screening cDNA libraries with a *BEL1* homeobox-specific probe under low stringency (A. S., Z. M., R. L. F., and G. W. H., unpublished data).

Molecular Analysis of the Mutant Alleles *bel1-1*, *bel1-2*, *bel1-3*, and *bel1-4*

To elucidate the molecular basis of the Bel1 mutant phenotype and confirm that we had identified the *BEL1* gene, we identified lesions in the two T-DNA-induced and two ethylmethylsulfonate (EMS)-induced alleles (see Experimental Procedures). In *bel1-2*, the T-DNA insertion resides within the first intron (Figures 1A and 1B). The T-DNA in *bel1-3* interrupts the gene in the 5′ untranslated leader (Figures 1A and 1B). The *bel1-1* and *bel1-4* mutations are C→T transitions resulting in translation termination codons at residues 116 (*bel1-4*) and 165 (*bel1-1*) (Figure 1B). The T-DNA insertions could affect RNA processing, while the stop codons in the EMS alleles could result in the premature termination of translation. Identification of the

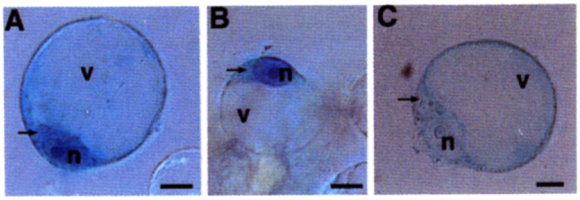

Figure 2. BEL1 Sequences Direct GUS Localization to the Nucleus

(A) BEL–GUS fusion. (B) VirD2–GUS positive control. (C) GUS only negative control. The nucleus (n) and cytoplasm (arrow) are appressed to the plasma membrane by the large central vacuole (v).

lesions in the mutations analyzed and complementation of *bel1-2* together indicate that we have isolated the wild-type *BEL1* genomic locus.

Nuclear Localization of *BEL1*

The presence of a DNA-binding domain in the BEL1 peptide implies that BEL1 functions as a transcription factor. Consistent with this prediction, a basic domain conforming to a bipartite nuclear localization motif (Chelsky et al., 1989) was identified in the region between residues 274–290 (Figure 1B). To determine whether sequences in BEL1 were sufficient to direct localization of a β-glucuronidase (*GUS*) reporter gene to the nucleus, a fusion of *BEL1* to *GUS* was transiently expressed as described in the Experimental Procedures. A blue precipitate indicating GUS activity was detected in the nucleus of protoplasts containing the *BEL1–GUS* fusion (Figure 2A) and the D2–*GUS* positive control (Figure 2B) (Howard et al., 1992). GUS did not localize to the nucleus in protoplasts containing only the *GUS* reporter gene (Figure 2C).

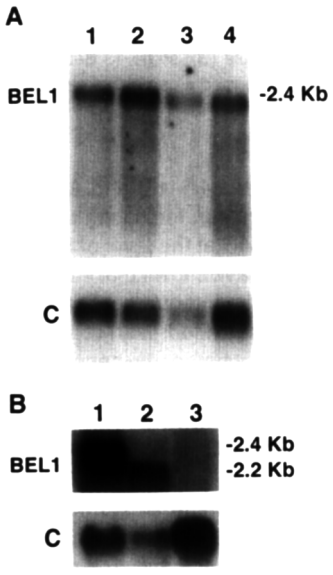

Figure 3. RNA Gel Blot Analysis of *BEL1* Expression

(A) Expression of *BEL1* in flower buds (stage 0–12) (lane 1), flowers (stage 13) (lane 2), siliques (lane 3), and leaves (lane 4). The 0.99 kb *ROC1* gene (Luppuner et al., 1994) was used as a control (C) for loading.

(B) Expression of *BEL1* in wild-type, *bel1-2*, and *bel1-3* stage 0–12 flower buds. The control (C) is a 1.35 kb 18s RNA gene (Jorgenson et al., 1987).

Expression of the *BEL1* Gene in Vegetative and Floral Organs

The pattern of *BEL1* RNA accumulation in vegetative and floral tissues shown in Figure 3A was determined using a labeled *BEL1* cDNA probe. A 2.4 kb *BEL1* RNA was detected in floral buds, open flowers, siliques, and seedlings (Figure 3A), as well as in leaves and roots (data not shown). We also determined the expression of *BEL1* in flowers of the two T-DNA alleles (Figure 3B). In flower buds of the *bel1-2* allele, the transcript detected is shorter, corresponding to a size of 2.2 kb (Figure 3B). The 2.4 kb transcript was not detected in RNA from floral buds (Figure 3B) or leaves (data not shown) of the *bel1-3* allele. These data indicate that *BEL1* RNA is expressed in both floral and vegetative tissues in wild-type plants.

In Situ Localization of *BEL1* RNA in Floral Tissues

To define precisely the spatial and temporal pattern of *BEL1* gene expression in flowers, we localized the *BEL1* RNA in situ using digoxigenin-labeled riboprobes (see Experimental Procedures). Hybridization signal appeared as a blue or brown precipitate. Figures 4A–4F illustrate the accumulation of *BEL1* RNA in wild-type flower and ovule development. To confirm that the pattern of expression observed with the *BEL1* antisense riboprobes was specific to the *BEL1* gene, sections of Bel1-3 flower buds were hybridized with the same probe on the same slides (Figures 4G and 4H), or slides were hybridized with a sense strand control (data not shown).

BEL1 RNA was first detected at stage 8 in ovules of wild-type flowers (Figure 4A). Within the ovules, *BEL1* RNA was present throughout the newly formed ovule primordium (Figure 4B). At stage 9, *BEL1* RNA accumulation was detected in the area between the nucellus and the funiculus (Figure 4C). This region corresponds to the position where the integuments will later form at stage 10. We detected *BEL1* RNA in the integument primordia (Figure 4D) and in the developing integuments (stage 11, Figure 4E). No *BEL1* RNA was detected in mutant ovule primordium (Figure 4G) or in the single integument-like structure in mutant ovules (Figure 4H). By stage 13, *BEL1* RNA was detected only in the chalazal region of the wild-type ovule (Figure 4F). We did not observe expression of *BEL1* in wild-type siliques or embryos up to the globular stage (data not shown), which corresponds to the time when the homeotic conversion in some Bel1-3 ovules becomes apparent (Modrusan et al., 1994). These data show that *BEL1* RNA accumulation in the flower is restricted to the ovule primordium and the integuments. Moreover, the early pattern of *BEL1* expression predicts the position where the inner and outer integuments will arise.

Expression of *AG* in Wild-Type and Bel1-3 Mutant Ovules

It has been proposed that the negative regulation of *AG* in the integuments by *BEL1* is required for normal ovule

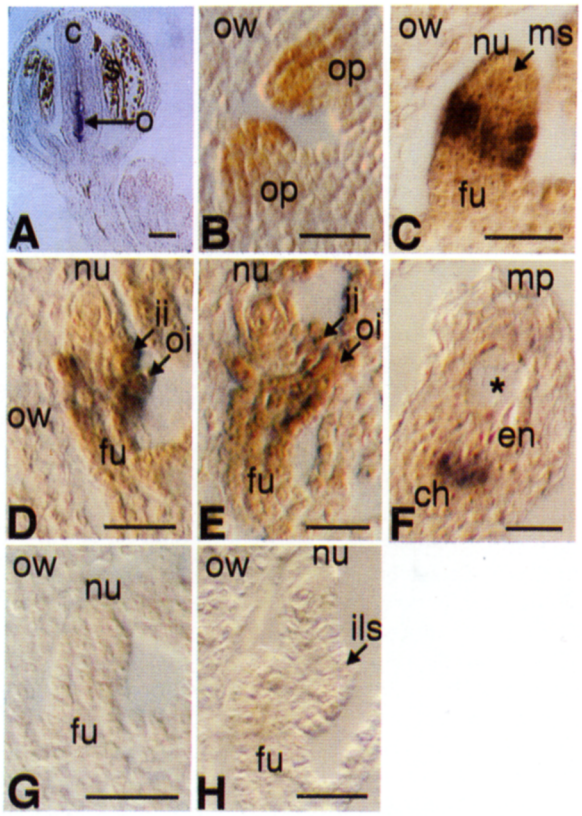

Figure 4. In Situ Localization of *BEL1* RNA in Wild-Type and Bel1-3 Ovules

All tissues were hybridized with a *BEL1* antisense riboprobe. Morphological features of flowers, ovary, and ovule are indicated as follows: stamen (s); carpel (c); ovule (o); ovary wall (ow); ovule primordium (op); megasporocyte (ms); funiculus (fu); nucellus (nu); inner integument (ii); outer integument (oi); endothelium (en); female gametophyte (asterisk); micropyle (mp); chalaza (ch), and integument-like structure (ils). Scale bar represents 10 μm, except for (A) where it represents 50 μm. Wild-type inflorescence (A); stage 8 wild-type ovule (B); stage 9 wild-type ovule (C); stage 10 wild-type ovule (D); stage 11 wild-type ovule (E); stage 13 wild-type ovule (F); stage 9 Bel1-3 ovule (G); stage 11 Bel1-3 ovule (H).

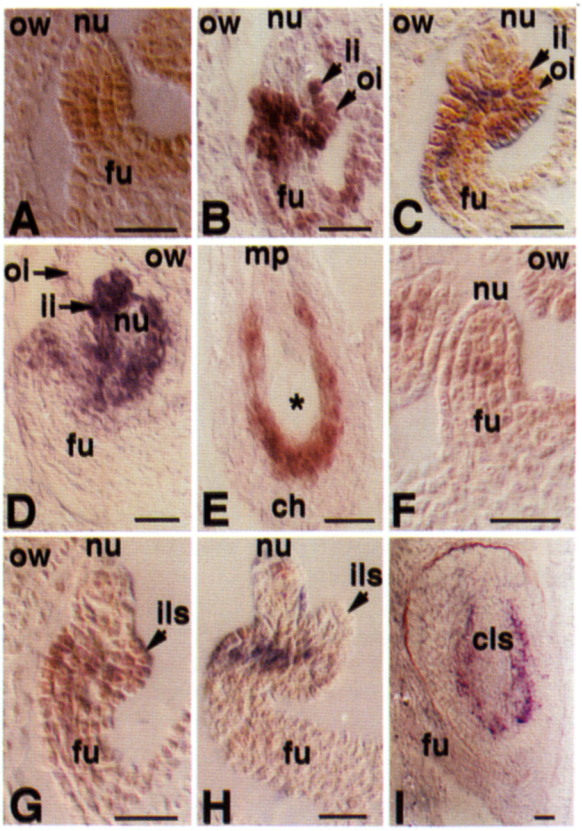

Figure 5. In Situ Localization of *AG* RNA in Wild-Type and Bel1-3 Mutant Ovules

All tissues were hybridized with the *AG* antisense riboprobe. Morphological features are indicated as in Figure 4. Scale bar represents 10 μm, except (I) where it represents 50 μm. Stage 9 wild-type ovule (A); stage 10 wild-type ovule (B); stage 11 wild-type ovule (C); stage 12 wild-type ovule (D); stage 13 wild-type ovule (E); stage 9 Bel1-3 ovule (F); stage 11 Bel1-3 ovule (G); stage 13 Bel1-3 ovule (H); stage 17 Bel1-3 ovule that has formed a carpel-like structure (cls) (I).

development. To test this model, we compared the patterns of *BEL1* and *AG* expression during wild-type ovule development. *AG* is expressed throughout the stamen and carpel primordia (Drews et al., 1991) and the initiating ovule primordium (data not shown). As shown in Figure 5, *AG* expression in the ovule at stage 9 was detected in the funiculus and the region where the integument primordia will form, but was not detected in the nucellus (Figure 5A). *AG* RNA was detected in the integument primordia (stage 10; Figure 5B) and continued to be expressed at high levels during stage 11, in both the inner and outer integuments (Figure 5C). By late stage 12, *AG* RNA was detected only in the inner integument layers (Figure 5D). In the mature stage 13 ovule, *AG* accumulation was restricted to the endothelium (Figure 5E) (see Bowman et al., 1991). *AG* RNA was not observed in postfertilization ovules up to the heart stage of embryo development (data not shown). During wild-type ovule development, the patterns of *BEL1* (Figures 4A–4F) and *AG* (Figures 5A–5E) gene expression overlap, indicating that *BEL1* does not negatively regulate *AG* at the level of RNA accumulation.

Previous results have shown that the *AG* gene is ectopically expressed in the integument-like structure of stage 14 Bel1 mutant ovules (Modrusan et al., 1994; Ray et al., 1994). To determine whether the pattern of *AG* expression is altered earlier in Bel1 ovules, we examined the expression of *AG* in Bel1-3 ovules throughout their ontogeny. The accumulation of *AG* RNA in Bel1-3 stage 9 ovule primordium was similar to the wild type (Figure 5F). *AG* RNA was detected in the single integument-like structure of Bel1-3 ovules (Figure 5G). At stage 13, *AG* RNA accumulation was detected primarily below the nucellus in Bel1-3 ovules (Figure 5H). During later stages of development (stages 14–17), persistent expression was observed in all Bel1-3 ovules, including those that had undergone a conversion to carpel development (Figure 5I). These data indicate that the pattern of *AG* expression in Bel1-3 ovules before integument initiation is similar to the pattern in wild-type ovules, suggesting that the absence of *BEL1* does

not affect distribution of *AG* RNA in ovule primordia before stage 12.

Discussion

The *BEL1* Gene Encodes a Homeodomain Transcription Factor

The *BEL1* gene encodes a protein that contains a homeodomain (Figure 1C). The homeodomain is a 61 amino acid DNA-binding motif that forms three α-helical regions; the third helix has been shown to be required for recognition of specific target DNA sequences (Gehring et al., 1994a, 1994b; Scott et al., 1989). Within the third, or recognition helix, BEL1 contains the four invariant amino acids conserved in all homeodomains (Figure 1C; W49, F50, N52, and R54) (reviewed by Gehring et al., 1994b; Scott et al., 1989).

Additional motifs characteristic of transcription factors are present in the BEL1 protein. We have shown that sequences within the *BEL1* coding region are sufficient to localize a BEL1–GUS fusion to the nucleus (Figure 2). The N-terminal region contains stretches of prolines and glutamines that have been implicated in activation of transcription in other systems (Figure 1B) (Gerber et al., 1994) as well as homopolymeric repeats (Lincoln et al., 1994). Helical wheel plots indicate that the region between amino acids 293–308 may form an amphipathic coiled-coil structure (Figure 1B) that could mediate interactions with other proteins. Several homeobox genes have been shown to act in combination with other transcription factors, and these interactions can modulate the specificity of homeobox gene activity (Gehring et al., 1994b). Finally, several acidic regions are found distributed throughout the predicted amino acid sequence (Figure 1B). Acidic domains are thought to function in transcriptional activation (Frankel and Kim, 1991). Taken together, these data indicate that BEL1 is a nuclear-localized homeodomain transcription factor.

Proximal–Distal Pattern Formation in the Ovule Primordium Involves the Spatial Regulation of *BEL1* Expression

In many organisms, pattern formation and organ identity is controlled, in part, by the action of homeobox genes (Gehring et al., 1994b; Krumlauf, 1994; Lawrence and Morata, 1994). Initially, the sequential activation of homeobox genes in the Drosophila embryo defines the anterior–posterior domains of morphogenetic boundaries in body segments in the larvae (Lawrence and Morata, 1994). Determination of segmental identity is then controlled by the action of the homeotic selector genes that are specifically localized within parasegments.

In contrast with animal systems, the functions of homeobox genes in plants are just beginning to be elucidated. Some homeobox genes, like *GLABRA2* (Rerie et al., 1994), may function in cellular morphogenesis. For other homeobox genes (i.e., *KN1* and some *KN1*-like genes), their patterns of RNA accumulation suggest that they respond to or may define patterning events in the shoot apical meristem (Jackson et al., 1994; Schneeberger et al., 1995). Ectopic expression of these genes in transgenic plants (Lincoln et al., 1994; Sinha et al., 1993) or in dominant mutants (Schneeberger et al., 1995; Smith et al., 1992) alters cell fates in leaves. However, until the phenotypes of loss-of-function mutations in these genes are determined, their functions in the shoot apex remain unclear.

Within the ovule, *BEL1* RNA accumulates in the region between the nucellus and the funiculus (see Figure 4C) and provides molecular evidence for the existence of a chalazal pattern domain (Schneitz et al., 1995). Thus, the *BEL1* homeobox gene responds to positional cues that establish the proximal–distal pattern of the ovule primordium. The loss-of-function phenotype of Bel mutant ovules indicates that the determination of integument initiation and organ identity is controlled by expression of the *BEL1* homeobox gene within the chalazal domain. Taken together, these results suggest that *BEL1* may function in a manner analogous to the Drosophila homeotic selector genes. That is, *BEL1* interprets positional information and controls morphogenesis of the integuments through the regulation of genes within the chalazal domain.

Within the pathway of ovule development, *BEL1* acts at intermediate stages of ovule development, downstream of ovule initiation and upstream of terminal differentiation. *BEL1* must then be subordinate to genes that are required for ovule initiation and ovule identity such as *FBP11* (Columbo et al., 1995) and *AP2* (Modrusan et al., 1994). Identification of the factor(s) that regulates *BEL1* expression will provide clues as to how positional information is specified and interpreted along the proximal–distal axis of the ovule.

BEL1 Regulates Development of Both the Inner and Outer Integuments

BEL1 RNA was detected in both integuments throughout their early development (see Figures 4C–4E) and not in the megasporocyte, functional megaspore, or female gametophyte. These data indicate that *BEL1* is not directly required for female gametophyte development that may require signals, nutrients, or mechanical support provided by normal integuments (Herr, 1995; Reiser and Fisher, 1993). In the absence of *BEL1* function, the inner integument fails to initiate, and the outer integument develops abnormally. Therefore, *BEL1* is directly required for inner integument initiation and outer integument identity.

The different roles that *BEL1* has in the inner and outer integument may reflect their disparate evolutionary origins. Although the exact origins of the integuments are subject to considerable debate (Friis and Endress, 1990; Herr, 1995), it is generally agreed that the outer and inner integuments were derived independently (Stebbins, 1974; Takhtadzhian, 1991). The inner integument evolved first in the gymnosperms, whereas outer integument developed later and is thought to have derived from a cupule or bract (Stebbins, 1974; Takhtadzhian, 1991). *BEL1* might represent an early inner integument specification gene that later came to be expressed in the cupule, shifting the cupule toward an integument-like fate. This is consistent with aspects of the Bel1 phenotype, whereby only inner integument initiation is affected and only the identity of the outer integument is altered. To show that *BEL1* is sufficient for

specification of the inner integument, it is necessary to examine the ovules of plants that ectopically express the *BEL1* gene.

BEL1 Does Not Negatively Regulate *AG* RNA Accumulation in the Integument Primordia

It has been suggested that one function of *BEL1* is to regulate *AG* expression in the ovule. Our in situ hybridization results indicate that *BEL1* does not antagonize *AG* at the level of RNA accumulation early in ovule development. We have shown that the domain of *AG* expression in the wild-type ovule overlaps substantially with that of *BEL1* (Figures 4 and 5). Furthermore, the early pattern of *AG* RNA accumulation is not affected by the absence of *BEL1* (Figure 5F). These results do not preclude the possibility that *BEL1* may regulate the activity of *AG* in the ovule, but suggest that the mechanism of regulation is not at the level of RNA accumulation.

Experimental Procedures

Mutant Lines and Growth Conditions

bel1-1 and *bel1-4* were gifts of Drs. C. Gasser and D. Preuss, respectively. Wild-type and mutant plants were grown in glass houses at the University of California, Berkeley under 16 hr light/8 hr dark photoperiods generated by supplemental lighting or under conditions described by Modrusan et al. (1994).

Screening of cDNA and Genomic Libraries

A genomic library of Bel1-2 mutant DNA was made in the λGT11 vector according to the instructions of the manufacturer (Promega) and screened with a T-DNA right border–specific probe. Plant sequences flanking the T-DNA border were used to screen wild-type genomic libraries from the Wassilewskja and Landsberg erecta (Ler) ecotypes (gifts of Dr. K. Feldmann and Dr. D. Jofuku, respectively). A 2.9 kb HindIII fragment from the wild-type region was used to screen floral-specific cDNA libraries generated from Ler plants (a gift from Dr. E. Meyerowitz). A 1.8 kb cDNA clone obtained from the flower library was used as a probe to screen a leaf-specific cDNA library made from Columbia ecotype plants (a gift of Dr. B. Staskawicz). Hybridization was performed under stringent conditions with ^{32}P random hexamer–labeled probes (Boehringer Mannheim).

Complementation of Bel1-2

Agrobacterium strain LBA4404, bearing plasmid MP90 and the kanamycin resistant binary vector pCGN1548 (McBride and Summerfelt, 1990), was utilized in a modified in planta transformation procedure (Bechtold et al., 1993; Katavic et al., 1994). To generate lines that segregated for both the wild-type transgene and the Bel1 phenotype, kanamycin resistant T1 plants were pollinated by a homozygous *bel1-2* plant. DNA from individual F2 progeny plants of this cross was isolated, and genotypes were determined by DNA gel blot analysis. A labeled BglII genomic fragment that detected a polymorphism between the *bel1-2* allele, wild-type gene, and wild-type transgene was used as a probe.

Sequencing of Wild-Type and Mutant Clones

Sequence of the 2.1 kb flower cDNA, 2.3 kb leaf cDNA, wild-type, *bel1-1*, and *bel1-4* genomic regions were obtained by the polymerase chain reaction (PCR) with Taq polymerase and resolved on an automated sequencer (Applied Biosystems, Incorporated). Sequence analysis was performed using the Genetics Computer Group software and the National Center for Biotechnology Information BLAST e-mail server.

The T-DNA–plant junction in *bel1-2* was sequenced using primers generated from the cDNA and intron sequences. The *bel1-3* genomic region was obtained by plasmid rescue of BglII-digested mutant DNA and sequenced to determine the T-DNA–plant junctions.

Nuclear Localization of BEL1-GUS Fusion

A 1.8 kb *BEL1* cDNA was introduced into the pRTL2–GUS vector as a translational fusion to the *GUS* reporter gene under the transcriptional control of the cauliflower mosaic virus 35S promoter (Restropo et al., 1990). Plant cell culture, electroporation, and GUS assays were performed as described by Howard et al. (1992). The protoplasts were examined and photographed with a Zeiss Axioskop equipped with Nomarski optics.

RNA Gel Blot Analysis

Total (Comai et al., 1992) or polyadenylated RNA (Poly A-Tract, Promega) from wild-type (Ler), *bel1-2*, and *bel1-3* mutant plants was isolated, electrophoresed, transferred to a membrane, hybridized, and washed under very stringent conditions (5°C–10°C below T_m). ^{32}P random hexamer–labeled *BEL1* probes (used in Figure 3A) spanned the region from nucleotides 490–2188. Antisense riboprobes (Figure 3B) were generated using Sp6 polymerase and EcoRI-digested pLR115, a subclone of a 2.1 kb flower cDNA in pGEM7Zf+ (Promega) that lacks ~200 bp of the 3′ end.

In Situ Hybridization

Tissues for in situ hybridization were prepared as described by Drews et al. (1991). Sections (8 μm) were adhered to Probe On Plus slides (Fisher). In situ hybridization was performed using riboprobes labeled with digoxigenin (Boehringer Mannheim). For *BEL1*, plasmid pLR115 was used to generate an antisense transcript. Sense and antisense transcripts of *AG* were obtained as described by Drews et al. (1991), except for the incorporation of digoxigenin-UTP. Hybridization and detection with nitrobluetetrazolium and X-phosphate were performed with a modified Genius protocol (G. Drews, personal communication). Sections were photographed on a Zeiss Axioskop using Nomarski and bright-field optics.

Image Processing

Photographs were scanned using a Scanmaker 600 color scanner (Microtek, Incorporated), and composite images were generated using Adobe Photoshop 3.0 (Adobe Systems). Figures were printed using a Phaser 440 dye sublimation printer (Tektronix, Incorporated).

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References

Barton, M.K., and Poethig, R.S. (1993). Formation of the shoot apical meristem in *Arabidopsis thaliana*: an analysis of development in the wild-type and in the shoot meristemless mutant. Development *119*, 823–831.

Bechtold, N., Ellis, J., and Pelletier, G. (1993). *In planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. CR Rev. Acad. Life Sci. *316*, 1194–1199.

Bowman, J.L., Drews, G.N., and Meyerowitz, E.M. (1991). Expression of the *Arabidopsis* floral homeotic gene *AGAMOUS* is restricted to specific cell types late in flower development. Plant Cell *3*, 749–758.

Chelsky, D., Ralph, R., and Jonak, G. (1989). Sequence requirements for synthetic peptide-mediated translocation to the nucleus. Mol. Cell. Biol. *9*, 2487–2492.

Coen, E.S., and Meyerowitz, E.M. (1991). The war of the whorls: genetic interactions controlling flower development. Nature *353*, 31–37.

Columbo, L., Franken, J., Koetje, E., Van Went, J., Dons, H.J.M., Angenent, G.C., and Van Tunen, A.J. (1995). The petunia MADS box gene *FBP11* determines ovule identity. Plant Cell *7*, in press.

Comai, L., Matsudaira, K.L., Heupel, R.C., Dietrich, R.A., and Harada, J.J. (1992). Expression of a Brassica-napus malate synthase gene in transgenic tomato plants during the transition from late embryogeny to germination. Plant Physiol. *98*, 53–61.

Drews, G.N., Bowman, J.L., and Meyerowitz, E.M. (1991). Negative regulation of the Arabidopsis homeotic gene *AGAMOUS* by the *APETALA2* product. Cell *65*, 991–1002.

Esau, K. (1960). Anatomy of Seed Plants (New York: John Wiley and Sons).

Frankel, A., and Kim, P. (1991). Modular structure of transcription factors: implications for gene regulation. Cell *65*, 717–719.

Friis, E.M., and Endress, P.K. (1990). Origin and evolution of angiosperm flowers. Adv. Bot. Res. *17*, 99–161.

Gehring, W., Qian, Y.-Q., Billeter, M., Furukubo-Tokunaga, K., Schier, A., Resendez-Perez, D., Affolter, M., Otting, G., and Wüthrich, K. (1994a). Homeodomain–DNA recognition. Cell *78*, 211–223.

Gehring, W.J., Affolter, M., and Bürglin, T. (1994b). Homeodomain proteins. Annu. Rev. Biochem. *63*, 487–526.

Gerber, H.P., Seipel, K., Georgiev, O., Hofferer, M., Hug, M., Rusconi, S., and Schaffner, W. (1994). Transcriptional activation modulated by homopolymeric glutamine and proline stretches. Science *263*, 808–811.

Goldberg, R.B., DePavia, G., and Yadegari, R. (1994). Plant embryogenesis: zygote to seed. Science *466*, 605–614.

Herr, J.M. (1995). The origin of the ovule. Am. J. Bot. *82*, 547–564.

Howard, E., Zupan, J., Citvosky, V., and Zambryski, P. (1992). The VirD2 protein of A. tumefaciens contains a C-terminal bipartite nuclear localization signal: implications for nuclear uptake of DNA in plant cells. Cell *68*, 109–118.

Jackson, D., Veit, B., and Hake, S. (1994). Expression of maize *KNOTTED1* related homeobox genes in the shoot apical meristem predicts patterns of morphogenesis in the vegetative shoot. Development *120*, 405–413.

Jorgenson, R.A., Cuellar, R.E., Thompson, W.F., and Kavanagh, T.A. (1987). Structure and variation in ribosomal RNA genes of pea: characterization of a cloned rDNA repeat and chromosomal rDNA variants. Plant Mol. Biol. *8*, 3–12.

Jürgens, G. (1995). Axis formation in plant embryogenesis: cues and clues. Cell *81*, 467–470.

Katavic, V., Haughn, G.W., Reed, D., Martin, M., and Kunst, L. (1994). *In planta* transformation of *Arabidopsis thaliana*. Mol. Gen. Genet. *245*, 363–370.

Kerstetter, R., Vollbrecht, E., Lowe, B., Veit, B., Yamaguchi, J., and Hake, S. (1994). Sequence analysis and expression patterns divide the maize *knotted1*-like homeobox genes into two classes. Plant Cell *6*, 1877–1887.

Korfhage, U., Trezzini, G.F., Meier, I., Halbrook, K., and Somssich, I.E. (1994). Plant homeodomain protein involved in transcriptional regulation of a pathogen defense-related gene. Plant Cell *6*, 695–708.

Krumlauf, R. (1994). *Hox* genes in vertebrate development. Cell *78*, 191–201.

Lawrence, P.A., and Morata, G. (1994). Homeobox genes: their function in Drosophila segmentation and pattern formation. Cell *78*, 181–189.

Lincoln, C., Long, J., Serikawa, K., and Hake, S. (1994). A *knotted*-like homeobox gene in *Arabidopsis* is expressed in the vegetative meristem and dramatically alters leaf morphology when overexpressed in transgenic plants. Plant Cell *6*, 1859–1876.

Luppuner, V., Chou, I., Scott, S., Ettinger, W., Theg, S., and Gasser, C. (1994). Cytosolic and chloroplast stromal forms of cyclophilin from *Arabidopsis thaliana*. J. Biol. Chem. *269*, 7863–7868.

Ma, H., McMullen, M.D., and Finer, J.J. (1994). Identification of a homoeobox containing gene with enhanced expression during soybean (Glycine max L.) somatic embryo development. Plant Mol. Biol. *24*, 465–473.

Mandel, A.M., Bowman, J.L., Kemplin, S.A., Ma, H., Meyerowitz, E.M., and Yanofsky, M.F. (1992). Manipulation of flower structure in transgenic tobacco. Cell *71*, 133–143.

Mansfield, S., Briarty, L., and Erni, S. (1991). Early embryogenesis in *Arabidopsis thaliana*. I. The mature embryo sac. Can. J. Bot. *69*, 447–460.

McBride, K.E., and Summerfelt, K.R. (1990). Improved binary vectors for *Agrobacterium*-mediated plant transformation. Plant Mol. Biol. *14*, 269–276.

Modrusan, Z., Reiser, L., Feldmann, K.A., Fischer, R.L., and Haughn, G.W. (1994). Homeotic transformation of ovules into carpel-like structures in *Arabidopsis*. Plant Cell *6*, 333–349.

Quaedvlieg, N., Dockx, J., Rook, F., Weisbeek, P., and Smeekens, S. (1995). The homeobox gene *ATH1* of *Arabidopsis* is depressed in the photomorphogenetic mutants *cop1* and *det1*. Plant Cell *7*, 117–129.

Ray, A., Robinson-Beers, K., Ray, S., Baker, S.C., Lang, J.D., Preuss, D., Milligan, S.B., and Gasser, C.S. (1994). *Arabidopsis* floral homeotic gene BELL (*BEL1*) controls ovule development through negative regulation of AGAMOUS gene (*AG*). Proc. Natl. Acad. Sci. USA *91*, 5761–5765.

Reiser, L., and Fisher, R. (1993). The ovule and the embryo sac. Plant Cell *5*, 1291–1301.

Rerie, G.W., Feldmann, K., and Marks, M.D. (1994). The *GLABRA2* gene encodes a homeodomain protein required for normal ovule development. Genes Dev. *8*, 1388–1399.

Restropo, M., Freed, D., and Carrington, J. (1990). Nuclear transport of plant potyviral proteins. Plant Cell *2*, 987–998.

Robinson-Beers, K., Pruitt, R., and Gasser, C. (1992). Ovule development in wild type *Arabidopsis* and two female sterile mutants. Plant Cell *4*, 1237–1249.

Ruberti, I., Sessa, G., Luccheti, S., and Morelli, G. (1991). A novel class of plant proteins containing a homeodomain with a closely linked leucine zipper. EMBO J. *10*, 1787–1791.

Schena, M., and Davis, R.W. (1992). HD-Zip proteins: members of an *Arabidopsis* homeodomain superfamily. Proc. Natl. Acad. Sci. USA *89*, 3894–3898.

Schneeberger, R.G., Becraft, P.W., Hake, S., and Freeling, M. (1995). Ectopic expression of the *KNOX* homeobox *rough sheath1* alters cell fate in the maize leaf. Genes Dev. *9*, 2292–2304.

Schneitz, K., Hulskamp, M., and Pruitt, R. (1995). Wild type ovule development in *Arabidopsis thaliana*: a light microscope study of cleared whole mount tissue. Plant J. *7*, 731–749.

Scott, M.P., Tamkun, J.W., and Hartzell, G.W. (1989). The structure and function of the homeodomian. Biochem. Biophys. Acta *989*, 25–48.

Slack, J.M.W. (1983). From Egg to Embryo: Determinative Events in Early Development (Cambridge: Cambridge University Press).

Sinha, N., Williams, R., and Hake, S. (1993). Overexpression of the maize homeobox gene *KNOTTED-1* causes a switch from determinate to indeterminate cell fates. Genes Dev. *7*, 787–795.

Smith, L., Greene, B., Veit, B., and Hake, S. (1992). A dominant mutation in the maize homeobox gene *KNOTTED-1* causes its ectopic expression in leaf cells with altered fates. Development *116*, 21–30.

Stebbins, G.L. (1974). Flowering Plants: Evolution above the Species Level (Cambridge, Massachusetts: Harvard University Press).

Takhtadzhian, A.L. (1991). Evolutionary Trends in Flowering Plants (New York: Columbia University Press).

Vollbrecht, E., Veit, B., Sinha, N., and Hake, S. (1991). The developmental gene *Knotted-1* is a member of a maize homeobox gene family. Nature *350*, 241–243.

Vollbrecht, E., Kerstetter, R., Lowe, B., Veit, B., and Hake, S. (1993). Homeobox genes in plant development: mutational and molecular analysis. In Evolutionary Conservation of Developmental Mechanisms, A.C. Spaulding, ed. (New York: Wiley–Liss), pp. 111–123.

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