receptor, and D. J. Loskutoff for human U-937 cells. Supported by NIH grant GM23562 and by a grant from the National Foundation for Cancer Research. K.W. was supported by a JSPS (Japan Society for the Promotion of Science) Postdoctoral Fellowships for Research Abroad.

15 January 1999; accepted 5 March 1999

ROUGH SHEATH2: A Myb Protein That Represses *knox* Homeobox Genes in Maize Lateral Organ Primordia

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The regulation of members of the *knotted1*-like homeobox (*knox*) gene family is required for the normal initiation and development of lateral organs. The maize *rough sheath2* (*rs2*) gene, which encodes a Myb-domain protein, is expressed in lateral organ primordia and their initials. Mutations in the *rs2* gene permit ectopic expression of *knox* genes in leaf and floral primordia, causing a variety of developmental defects. Ectopic KNOX protein accumulation in *rs2* mutants occurs in a subset of the normal *rs2*-expressing cells. This variegated accumulation of KNOX proteins in *rs2* mutants suggests that *rs2* represses *knox* expression through epigenetic means.

Regulation of *knox* gene expression determines the emergence of lateral organs from shoot meristems. In maize, the KNOTTED-1 (KN1) homeodomain protein accumulates in cells of the shoot apex and maintains the meristematic properties of the cells (*1*). Recruitment of leaf founder cells (and the down-regulation of *kn1*) begins at a single site on the flank of the shoot apex and continues laterally around the circumference of the apex (*1*, *2*). Other *knox* family members, including *rough sheath1* (*rs1*), are also implicated in leaf initiation and patterning (*3*–*5*).

The *knox* gene products are absent in normal leaf and floral primordia (*1*, *3*, *6*). Ectopic *knox* expression during organogenesis interferes with organ determination and cell differentiation along the adaxial/abaxial and proximodistal axes (*5*, *7*, *8*). In maize, dominant mutations in *knox* genes cause the distal displacement of sheath, auricle, and ligule tissues (*5*). In dicot species, overexpression of *knox* genes results in the development of filamentous and lobed leaves and in the formation of ectopic meristems (*8*).

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In maize, recessive mutations that affect *knox* gene repression have also been identified. The *narrow sheath* and *leafbladeless1* mutations affect *kn1* down-regulation at leaf initiation, resulting in deletion of the leaf margins and development of radially symmetrical, abaxialized leaves, respectively (*9*). Mutations in *rs2* result in proximodistal patterning defects that are due to *rs1* and *kn1* expression in leaf primordia (*10*). Recessive mutations in the *PHANTASTICA* (*PHAN*) gene of *Antirrhinum*, which encodes a Myb-domain protein, exhibit phenotypes that resemble the phenotypes of these maize mutants (*6*, *11*). Therefore, we investigated the *phan* homolog from maize (*12*).

The maize *phan* homolog encodes a 370–amino acid protein with a 106–amino acid NH_2-terminal Myb domain consisting of two Myb-like repeats (Fig. 1A). The Myb domain and COOH-terminus share a high degree of amino acid identity with PHAN proteins from *Antirrhinum* and *Arabidopsis*. The DNA recognition helices of PHAN share little homology with the other large class of plant Myb proteins (*13*), suggesting that the PHAN proteins regulate a different class of target genes. However, the PHAN proteins do not contain motifs that suggest a direct transcriptional function. A single intron in the 5′ untranslated region (UTR), ~50 nucleotides upstream of the translation initiation codon, indicates a structural relation between the *Antirrhinum* and maize *phan* genes (Fig. 1B) (*12*).

The maize *phan* homolog was mapped to a region on chromosome arm 1S that contains a potential *knox* gene regulator, the *rs2* gene (*14*). A comparison of the structure of the *phan* locus in wild type and in three mutant alleles of *rs2* confirmed that *rs2* is the maize homolog of *phan* (Fig. 1B) (*15*). The *rs2* mutations cause leaf and floral phenotypic alterations analogous to the phenotypes induced by mutations that alter the regulation of *knox* genes during lateral organ initiation or development (*5*, *10*).

We compared the pattern of KNOX protein expression in wild-type and *rs2* mutant apices by immunolocalization with an antibody specific to KNOX proteins, including KN1 and RS1 (*10*, *16*). KNOX proteins accumulated in the shoot apex and stem of wild-type plants but were absent at leaf initiation sites on the apex and in leaf primordia (*1*) (Fig. 2, A and C). In *rs2* mutants, KNOX proteins accumulated normally in the meristem and stem, but they also accumulated at the base of leaf primordia and near major lateral veins in the leaf (Fig. 2, B and D). The ectopic accumulation of KNOX protein in patches with sharp lateral boundaries suggests that the leaves were clonal mosaics of *knox*+ and *knox*– sectors (Fig. 2B). Sectors expressing KNOX proteins varied among leaves and did not correlate with normal developmental domains. The down-regulation of *knox* expression at the initial site of founder cell recruitment near the center of the new leaf occurred normally in *rs2* mutants (Fig. 2D), although the number of founder cells that were recruited laterally was variably reduced.

These patterns of KNOX protein accumulation were compared to the distribution of *rs2* and *kn1* transcripts (*17*). In wild-type apices, *rs2* transcripts accumulated throughout the P1 leaf primordium, but in later stages of leaf development (P2 through P5), *rs2* expression became more restricted to the major vascular bundles and leaf margins (Fig. 3A). *rs2* was not expressed in the meristem, but expression was observed late during founder cell recruitment, at the transition from the P0 to the P1 stage. In contrast, *kn1* was expressed in meristematic cells of the shoot apex but was absent in early leaf founder cells (early P0 stage) (Fig. 3B). No *rs2* transcripts were detected in the reference allele of *rs2* (*rs2-R*) mutant shoot apices (Fig. 3C), but the *kn1* expression pattern in the meristem was unaltered. As in vegetative apices, *kn1* expression was limited to meristematic cells in flowers and was down-regulated in floral organ primordia and their initials (Fig. 3, E and H). *rs2* transcripts accumulated relatively early in founder cells of floral organ primordia (Fig. 3, D and G), and *rs2* expression per-

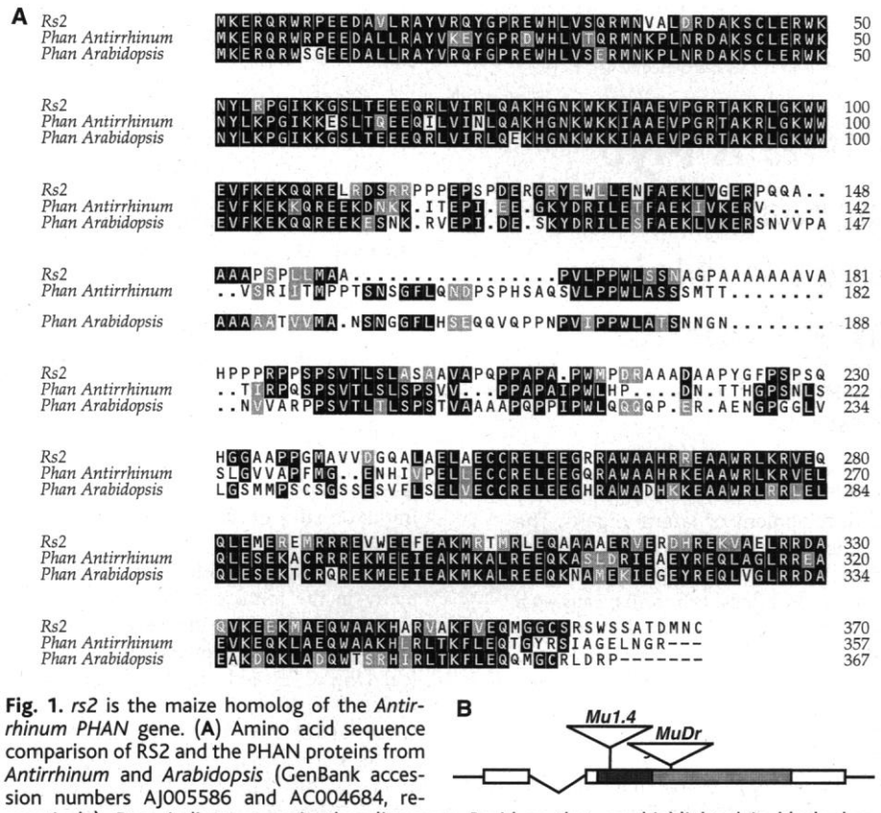

Fig. 1. *rs2* is the maize homolog of the *Antirrhinum PHAN* gene. (**A**) Amino acid sequence comparison of RS2 and the PHAN proteins from *Antirrhinum* and *Arabidopsis* (GenBank accession numbers AJ005586 and AC004684, respectively). Dots indicate gaps in the alignment. Residues that are highlighted in black show identity, and conserved residues are highlighted in gray. (**B**) Schematic diagram of the *rs2* locus. White boxes, untranslated exons; gray box, translated region; dark gray box, the Myb domain. Triangles mark the positions of the *Mu1.4* and *MuDR* transposable elements in the *rs2-mum1* and *rs2-mum2* alleles, respectively. *rs2-R* corresponds to a deletion of the Myb domain and the 5′ region.

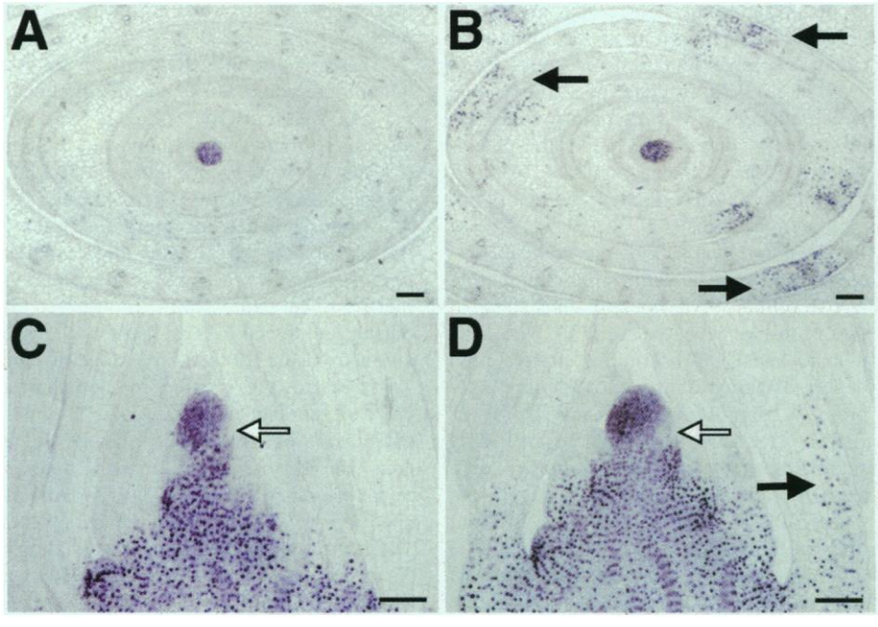

Fig. 2. *rs2* is required to repress *knox* gene expression in lateral organs. Immunolocalization of KNOX protein in (**A** and **C**) wild-type and (**B** and **D**) *rs2-R* shoot apices. The transverse (A and B) and longitudinal (C and D) sections are shown. Solid arrows denote some sectors that ectopically accumulate KNOX proteins. Open arrows mark the site on the flank of the shoot apex at which founder cell recruitment has initiated and KN1 protein is no longer present. Scale bar, 100 μm.

sisted in young floral organ primordia, including both pistil and stamen (Fig. 3G). No *rs2* expression was observed in silk (styles), but *rs2* transcripts were detected during the later stages of stamen development in the tassel. The *rs2* expression pattern in normal leaf and floral organ primordia is consistent with the *rs2* mutant phenotype and with the pattern of ectopic KNOX protein accumulation in *rs2* mutant apices. The differentiation of auricle and sheathlike tissue occurs in sectors along the major lateral veins in the blade region of *rs2* mutant leaves. In addition, *rs2* mutations frequently affect founder cell recruitment and development of the leaf margins (*10*).

The amino acid sequence conservation between RS2 and PHAN and the resemblance of the *phan* mutant phenotype to that of maize mutants defective in *knox* gene regulators (*6, 9–11*) suggest that RS2 and PHAN have analogous functions. Consistent with this, expression of the *Antirrhinum* homeobox gene *SHOOTMERISTEMLESS* (*AmSTM*) (*6*) is restricted to meristematic tissues in wild-type *Antirrhinum* but was observed in corollas and young leaves of the *phan* mutant (Fig. 4) (*18*). However, unlike *rs2*, *phan* mutants exhibit adaxial/abaxial polarity defects (*11*). This suggests that *knox* expression in leaves has different effects on leaf development in maize and *Antirrhinum*. KNOX proteins delay the transition from cell proliferation to differentiation (*5, 8*). The spatial pattern of this transition in leaves differs among plant species, resulting in different leaf morphologies. In maize, this pattern is basipetal, such that ectopic *knox* expression results in a distal displacement of features (*5*). The development of abaxial features on the adaxial leaf surface of *phan* mutant leaves suggests that this transition in *Antirrhinum* is deferred along both the proximodistal and adaxial/abaxial axes. Alternatively, *PHAN* may affect the regulation of additional target genes, or aspects of the *rs2* gene function may be masked by the action of other maize genes with partially redundant functions.

Our observations suggest that *rs2* and *PHAN* act directly or indirectly to maintain *knox* genes in an "off" state, preventing their expression in leaf and floral primordia and their founder cells. Thus, the *rs2*- and *PHAN*-encoded Myb-domain proteins have a function that is analogous to the *CURLY LEAF* gene of *Arabidopsis*, which encodes a Polycomb-like factor that suppresses the expression of floral homeotic genes in vegetative parts of the plant (*19*). In organisms such as *Drosophila* and yeast, key homeobox genes are spatially regulated by chromatin remodeling factors that confer "cellular memory" (*20*). The patchiness of

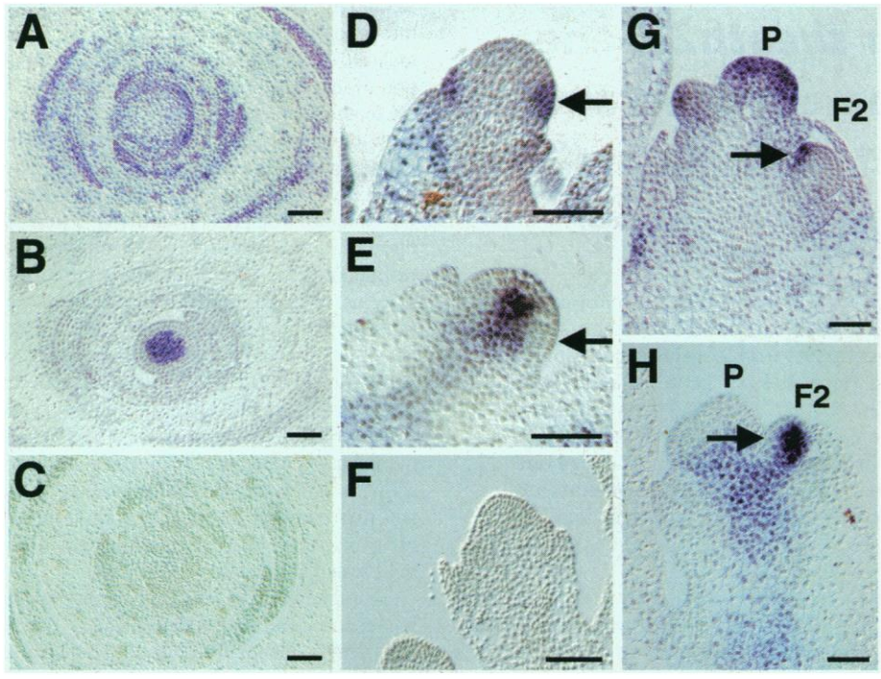

Fig. 3. *rs2* and *kn1* are expressed in mutually exclusive domains in vegetative and floral apices. (**A** and **B**) In situ hybridization of wild-type shoot apices with probes for *rs2* (A) or *kn1* (B). (**C**) *rs2-R* shoot apex hybridized with a *rs2*-specific probe. (**D** through **F**) Spikelets with young glume primordia probed with *rs2* (D), *kn1* (E), or a sense probe derived from *rs2* (F). Arrow in (D) marks the accumulation of *rs2* transcripts in the P0 initials of the inner and outer lemmas. Arrow in (E) marks the absence of *kn1* transcripts in the corresponding positions. (**G** and **H**) Comparable sections through a spikelet that has initiated the lower floret, showing the accumulation of *rs2* (G) but not of *kn1* (H) in the pistil. The arrows in (G) and (H) mark the adaxial cells on the lower floret that no longer express *kn1* but that do express *rs2*. P, pistil primordium; F2, secondary or lower floret. Scale bar, 50 μm.

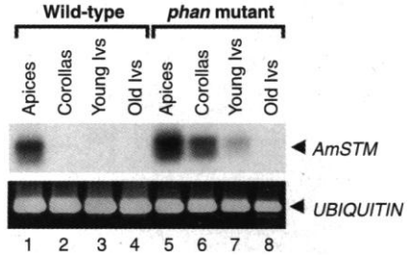

Fig. 4. Ectopic *AmSTM* expression in lateral organs of the *Antirrhinum phan* mutant. Reverse transcriptase–PCR analysis of the expression levels of the *knox* gene, *AmSTM*, and the *Antirrhinum UBIQUITIN* gene in vegetative shoot apices and lateral organs (lanes 2 through 4 and 6 through 8) of either wild-type (lanes 1 through 4) or *phan*-mutant (lanes 5 through 8) plants. lvs, leaves.

phenotypic effects in *rs2* mutant leaves, together with the apparent clonal sectors of KNOX protein accumulation, is suggestive of an imperfect silencing of *knox* gene activity that is clonally propagated during primordium development. Although the *rs2* alleles that we analyzed are null alleles, *rs2* mutant leaves appear as mosaics of normal and abnormal cell differentiation, and KNOX proteins accumulate only in a variable subset of the normal *rs2*-expressing cells. In addition, the *rs2* and *phan* mutants exhibit phenotypes that are dependent on temperature and on genetic background (*6, 10, 11*). These observations suggest that *rs2* acts on *knox* genes as an epigenetic regulator (*21*).

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12. A cDNA library derived from vegetative apices of maize inbred B73 (provided by S. Hake, U.S. Department of Agriculture Plant Gene Expression Center, Albany, CA) was screened using the region of the *PHAN* cDNA encoding the Myb domain as a probe. Two cDNA clones were isolated from 1×10^6 to 2×10^6 plaques screened. One cDNA was made up of 1681 base pairs (bp) and encodes a full-length protein. Amino acid sequences of RS2 and PHAN proteins from *Antirrhinum* and *Arabidopsis* were aligned using Pileup (Genetics Computer Group, University of Wisconsin, Madison, WI) with a gap weight of 5 and a length weight of 0.1. An 11.5-kb Bam HI genomic clone was isolated from a size-selected genomic library. Introns were identified by restriction analysis of the genomic and cDNA clones, followed by polymerase chain reaction (PCR) and sequence analysis. An intron of ~2.4 kb is present in the 5′ UTR.
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14. The maize *phan* locus was mapped with recombinant inbred populations [B. Burr, F. Burr, K. Thompson, M. Albertson, C. Stuber, *Genetics* **118**, 519 (1988)] to a position that is three map units distal to *npi598* on chromosome arm 1S.
15. Additional *rs2* alleles were isolated by transposon tagging. Plants that are homozygous for the *rs2-R* allele were crossed as males to stocks carrying active *Mutator* transposons. New *rs2* mutants were identified in F_1 screens at a frequency of 1 in ~12,000 plants. The nature of the mutation in the *rs2-mum1* and *rs2-mum2* alleles was determined by restriction analysis.
16. Shoot apices of 2-week-old *rs2* and wild-type sibling seedlings were fixed and embedded as described [D. Jackson, in *Molecular Plant Pathology: A Practical Approach*, D. Bowles, S. Gurr, M. McPherson, Eds. (Oxford Univ. Press, Oxford, 1991), pp. 163–174]. Immunohistochemistry with a KNOX-specific polyclonal antibody (provided by M. Freeling, University of Califomia, Berkeley) was performed as described (*1, 9*).
17. Tissue sections were prepared and hybridized as described (*3, 16*). Digoxigenin-labeled probes were prepared by in vitro transcription (Boehringer Mannheim), according to the manufacturer. *kn1*-specific probes have been described (*3*) and were used at a concentration of 0.5 ng μl^{-1} kb^{-1} probe complexity. Subclones covering different regions of the *rs2* cDNA that gave specific Southern (DNA blot) hybridization signals under normal stringent conditions were used to generate *rs2*-specific probes. These probes were used at a concentration of 5 ng μl^{-1} kb^{-1} probe complexity.
18. Five micrograms of total RNA from vegetative shoot apices, young floral corollas (<5 mm), young leaves (<5 mm), and fully expanded leaves of either wild type or *phan*-607 mutants (*6*) were used in cDNA synthesis with an oligo(dT) primer. One percent of each cDNA pool was used as a template in PCR reactions with *AmSTM*-specific primers capable of amplifying 419 bp of cDNA surrounding the position of the 405-bp intron (*6*). Specific PCR products of 419 bp were detected by Southern (DNA blot) hybridization with an *AmSTM* probe. Control amplification with primers specific for the *Antirrhinum UBIQUITIN* gene (X67957) under the same conditions indicated that similar amounts of cDNA were present in all samples.
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22. The authors thank M. Tsiantis, R. Schneeberger, M. Freeling, and J. Langdale for discussions of their independent cloning of *rs2*; our colleagues at Yale University and Cold Spring Harbor Laboratory for useful discussions; and T. Mulligan and J. Truglio for help in the field and in the laboratory. P.W.B. was supported by a grant from NIH to M. Freeling. This research was supported by an award from USDA to T.N.

3 November 1998; accepted 3 March 1999