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.

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 PHAN-TASTICA (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 NH2-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 target genes (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, knl 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-
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 bract, showing the glume primordia probed with rs2-R shoot apex hybridized with a rs2-specific probe. (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) Arrow in (H) marks the accumulation of \( \text{kn1} \) (D) and the absence of \( \text{kn1} \) (E) but not of \( \text{kn1} \) (F). Arrow in \( \text{kn1} \) (G) and (H) mark the adaxial cells on the lower bract that no longer express \( \text{kn1} \) but that do express rs2. P, pistil primordium; F2, secondary or lower bract. Scale bar, 50 \( \mu \)m.

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.

References and Notes

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 \( 1 \times 10^5 \text{ to } 2 \times 10^5 \) 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. Intonos were identified by restriction analysis of the genomic and CDNA clones, followed by polymerase chain reaction (PCR) and sequence analysis. An intron of \( \sim 2.4 \) kb is present in the 5′ UTR.
14. The maize phan locus was mapped with recombiant inbred populations [B. Burr, F. Burr, K. Thompson, M. Albertson, C. Stubbs, Genetics 118, 519 (1988)] to a position that is three map units distal to npi798 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 F2 screens at a frequency of 1 in \( \sim 12,000 \) 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. Gutt, M. McPherson, Eds. (Oxford Univ. Press, Oxford, 1991), pp. 163–174]. Immunohistochemistry with a KNOX-specific polyclonal antibody (provided by M. Freeling, University of California, 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 \( \mu \text{l}^{-1} \) 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 \( \mu \text{l}^{-1} \) 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 AntSTM-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 AntSTM 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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