Ectopic expression of the knox homeo box gene rough sheath1 alters cell fate in the maize leaf

Richard G. Schneeberger,1,4 Philip W. Becraft,1,3,4 Sarah Hake,1,2 and Michael Freeling1,5

1Department of Plant Biology, University of California, Berkeley, California 94720 USA; 2Plant Gene Expression Center, USDA-ARS, Albany, California 94710 USA

Rough sheath1 (Rsl) is a dominant mutation that alters cell fate and causes unregulated cell division and expansion in the maize leaf. A knox (Knl-like-homeo box gene) sequence closely linked to the Rsl-O mutation was cloned and shown by transposon mutagenesis to encode the rsl gene. The deduced amino acid sequence of the RS1 protein is highly similar to KN1 in the homeo domain but contains a unique amino-terminal region. rsl is expressed in the shoot apical meristem in a circular pattern preceding leaf initiation, but is not detectable in leaf primordia or mature leaves in normal plants. Rings of rsl expression subtend leaf insertion sites in the shoot, and lateral organ primordia in inflorescence and floral meristems. The timing and position of rsl expression in meristems suggests a possible role for rsl in patterning the placement of lateral organs along the axis of the shoot. In contrast to wild type, rsl is expressed in early leaf primordia of Rsl mutant plants, suggesting that ectopic expression causes the mutant phenotype. Ectopic expression in Rsl-O plants suggests the lingular region is more competent to respond to rsl than blade or sheath tissues.

[Key Words: Maize; plant; homeo domain; rough sheath1; leaf; development]

Received June 1, 1995; revised version accepted August 7, 1995.

Maize leaves consist of a basal portion called the sheath, and a broad, distal portion called the blade. A tissue fringe called the ligule, and two triangular structures termed auricles form a hinge-like structure that defines the precise boundary between the blade and sheath. (Fig. 1A,D). The normal regional organization of the blade sheath boundary is disrupted by at least six dominant mutations including rough sheath1 (rs1) and knotted1 (kn1). Cells that normally acquire a blade fate are transformed into sheath-like cells [Freeling 1992]. Rs1 mutations cause a proliferation of tissue at the blade/sheath boundary [Figure 1B,E]. The epidermis of the affected region has sheath/auricle attributes and is associated with ectopic or displaced ligule. Internal cell layers are characterized by a proliferation of mesophyll cells, abnormal cell shape, and defects in vein morphology/arrangement in the affected region of the leaf [Becraft and Freeling 1994]. Similarly, Kn1 mutations cause finger-like projections (knots) to form over lateral veins on the leaf blade [Fig. 1C,F] [Gelinas et al. 1969; Freeling and Hake 1985]. The knots are thought to form as a result of delayed maturation and prolonged growth of this tissue. The tissue surrounding lateral veins of Kn1 mutants has histological and morphological attributes of sheath and auricle tissue [Becraft and Freeling 1989; Sinha and Hake 1994]. The ligule is often displaced, or forms ectopically, along lateral veins in the blade at new blade–sheath junctions. Thus Rs1 and Kn1 mutations have similar phenotypes involving prolonged growth and altered cell fates. The mutants are also genetically similar; both are neomorphic and act noncell-autonomously [Freeling and Hake 1985; Hake and Freeling 1986; Sinha and Hake 1990; Becraft and Freeling 1994].

kn1 was cloned by transposon tagging [Hake et al. 1989] and was the first plant gene shown to contain a homeo box [Vollbrecht et al. 1991]. The homeo box is a 183-bp nucleotide sequence encoding a 61-amino-acid [64 in KN1] motif called the homeo domain. The homeo domain was first identified in the Drosophila Antennapedia and Ultrabithorax genes as a sequence with similarity to the helix-turn-helix motif of prokaryotic repressor proteins [Laughon and Scott 1984; Gehring et al. 1994]. Several homeo domains have been experimentally shown to function as sequence-specific DNA-binding
proteins that contain a helix-turn-helix motif, and act as transcriptional regulators (for review, see Levine and Hoey 1988; Hayashi and Scott 1990; Gehring et al. 1994). The homeo domain motif has subsequently been identified as a common sequence element in key developmental regulatory genes in yeast, fungi and many animal species (for review, see Scott et al. 1989; Gehring et al. 1994; Lawrence and Morata 1994). BX-C and ANT-C complex homologs have been found in all insect species examined as well as crustaceans, nematodes, chordates, and mammals, suggesting an evolutionary conservation of developmental mechanism (for review, see McGinnis and Krumlauf 1992; Kenyon 1994). Gene complexes similar to the BX-C and ANT-C have not been identified in plants to date (Kerstetter et al. 1994). Plant homeo box genes, however, appear to function as developmental switches, suggesting that the homeo domain has been used as a developmental regulator in both plants and metazoans (Rerie et al. 1994; Quaedvlieg et al. 1995).

The phenotypic and genetic similarities between Rs1 and Kn1 mutants suggested that they were related genes. We show that rs1 contains a homeo box with high sequence similarity to kn1, and that Rs1 mutants ectopically express rs1 transcript in leaf primordia at an early point in development, coincident with the formation of the blade–sheath boundary.

**Results**

**Identification and cloning of a homeo box-hybridizing genomic fragment that cosegregates with the Rs1-O mutant phenotype**

DNA was analyzed from mutant and normal individuals of families segregating 1:1 for the Rs1-O mutation to identify a homeo box-containing genomic fragment linked to the rs1 gene. The DNA was digested with BamHI, EcoRI, HindIII, or SstI, gel blotted, and the membranes probed at low stringency with a 247-bp kn1 homeo box probe (Vollbrecht et al. 1991). Approximately 14 hybridizing fragments were detectable in genomic DNA from the inbred line B73 (Fig. 2A).

A 2-kb SstI fragment was identified in all Rs1-O mutant samples but not in wild types (Fig. 2A). Among 86 individuals from three different segregating families, all the mutants and none of the normal plants contained the 2-kb SstI fragment, indicating that this genomic fragment was genetically linked to rs1 within 1.2 map units.

A subgenomic SstI library was constructed and screened with the kn1 homeo box probe. Clone Pb1 contained an insert of the desired 2-kb size and was used as a hybridization probe to an SstI-digested genomic DNA gel blot from a family segregating 1:1 for Rs1-O and wild-type alleles. The probe hybridized to the expected 2-kb fragment in all the mutant individuals and not in wild types. The probe also recognized a 1.9-kb fragment in homoyzgous wild-type and heterozygous mutant plants (Fig. 2B). At high stringency, no other hybridization was detected, demonstrating that the cloned insert corresponds to the rs1-linked fragment and represents a unique genomic sequence.

**Transposon mutagenesis confirms that a cloned, cosegregating, genomic fragment is the rs1 gene**

The tight linkage between rs1 and a kn1-related genomic sequence, together with the phenotypic and genetic similarities between Rs1 and Kn1 mutants provided circumstantial evidence that the cloned fragment was the rs1 gene. This hypothesis was verified with transposon mutagenesis. Maize stocks were generated that were homozygous Rs1-O and harbored active Mutator transposable elements. These stocks were crossed as females by wild-type plants to produce an F1 population that was homozygous Rs1-O [Rs1-O/+ ]. As such, most displayed a mutant phenotype, except for rare instances where the mutant function of the Rs1-O allele was abolished by a second mutation. Approximately 90,000 individuals were screened and 19 different families contained plants that appeared normal or had weak mutant phenotypes. Sixteen families contained single normal individuals, two had two normal plants, and one family had 10 normal plants. Normal plants within a family may have arisen from a single event, such as an ear sector, whereas normal plants in different families represent independent events.

DNA from Rs1-O progenitors, wild-type parents, normal progeny, and their mutant siblings was analyzed by

---

**Figure 1.** Comparison of the phenotypes of the ligular region of wild-type (A,D), Rs1-O mutant (B,E), and Kn1-O (C,F) mutant leaves. The leaves shown in A–C are viewed from the abaxial surface. (a) Auricle; (b) blade; and (s) sheath. (D–F) Diagram of an adaxial view of the three phenotypes. Both Rs1-O and Kn1-O leaves show a proliferation of sheath-like tissue that would normally have been blade (indicated by dark cross hatching in E and F). (Open regions) Blade; (light cross hatching) auricle/ligule; (closed regions) sheath.
increased molecular weight of the Rs1-O fragment in Rs1-Or324, r90, r122, and r312 revertants [see asterisks in Fig. 3]. In addition, revertant Rs1-Or56 [r56] contained a deletion of the Rs1-O allele as evidenced by lack of the Rs1-O band in multiple digests [Fig. 3, lane 7]. Probes spanning the entire length of the rs1 gene detect only the normal tester allele, indicating that the entire rs1 locus has been deleted (R. Scheeberger, unpubl.). Genetic crosses of r56 as a male resulted in the recovery of only the normal tester allele in 20 plants examined, indicating that the r56 allele is not transmitted through the male gametophyte. Examination of pollen from r56 heterozygotes shows ~50% pollen death, supporting the conclusion that the deletion is lethal to the male gametophyte. The correlation of both DNA insertion events

DNA gel blotting (Fig. 3). DNA gel blots prepared with either HindIII (lanes 1–9) or SstI (lanes 10–15) digested DNA were probed with pB1 or p845P [a 480-bp PstI fragment from the 5’ end of the gene; Fig. 4]. Seventeen of the 19 samples showed a detectable difference in the migration of the Rs1-O fragment indicating that an alteration in the DNA within this fragment was associated with the loss of the Rs1-O mutant phenotype. Figure 3 shows four independent insertion events as evidenced by

Figure 2. Cosegregation of a kn1-like homeo box sequence with the Rs1-O mutant phenotype. (A) A DNA gel blot of SstI-digested genomic DNA probed at low stringency with the kn1 homeobox, showing a band that cosegregates with the Rs1-O mutant phenotype. The arrow denotes the 2-kb fragment that was present in all mutants but not in wild-types. (B) A blot of SstI-digested DNA samples from a family segregating for Rs1-O, probed at high stringency with the 2-kb SstI insert. The insert represents the correct genomic fragment that cosegregates with Rs1-O and is a unique sequence. The probe hybridizes to the 2-kb fragment in all mutants and to a 1.9-kb fragment associated with the wild-type allele that is present both in the heterozygous mutants and in the homozygous wild types.

Figure 3. A genomic DNA gel blot showing that insertion into the Rs1-O linked fragment is associated with loss of the Rs1-O phenotype. Genomic DNA was digested with either HindIII or SstI and the blots were probed with either pB1 or p845P [Fig. 4]. (Lanes 1–4) HindIII-digested female and male progenitor DNA (lanes 1 and 4, respectively), nonrevertant [Rs1-O phenotype] sibling [lane 2] and partial revertant Rs1-Or349 [lane 3]. Hybridization with probe pB1 [Fig. 4] detects a 13-kb Rs1-O progenitor fragment. The revertant individual contains a 15-kb fragment indicating that an ~2-kb insertion was associated with the reversion event. (Lanes 5–15) DNA gel blot analysis of four full revertants Rs1-Or56, r90, r122, and r312. Each revertant is compared to the Rs1-O homozygous female parent and a nonrevertant sibling from the same family. DNA in lanes 5–9 is digested with HindIII and in lanes 10–15 is digested with SstI. Hybridization probes; lanes 5–12, p845P; lanes 13–15, pB1. In each case the Rs1-O revertant samples show an altered or missing [Rs1-O r56] rs1 hybridizing fragment not shared by a nonrevertant sibling from the same family. The genotypes of each sample are shown above the lanes. Asterisks show altered Rs1-O revertant polymorphisms.
Supporting evidence that the cloned homeo box sequence encodes the \( rsl \) gene comes from the isolation of the \( Rsl-1025 \) allele. This allele arose independently in a stock containing \( Mutator \) transposons. The \( Rsl-1025 \) mutant phenotype is very similar to \( Rsl-1 \) and segregates with the \( rsl \) gene on chromosome 7S [Becraft and Freeling 1994]. A \( \lambda \) genomic clone of the \( Rsl-1025 \) allele was isolated and shown to contain a \( Mu6/7 \) element [Chandler and Hardeman 1992] inserted into the third intron of the \( rsl \) gene, confirming that this sequence is responsible for the \( Rough \) \( sheath \) 1 phenotype [Fig. 4].

The \( rsl \) gene encodes a homeo domain protein that is highly similar to the product of \( knotted1 \)

A maize ear primordium cDNA library was screened with the \( kn1 \) homeo box probe. Four cDNA clones were isolated that contain \( rsl \) sequence obtained from the 2-kb \( SstI \) \( rsl \) genomic clone \( pB1 \). The longest clone contained \( \approx 800 \) bp of sequence that matched exactly the sequence obtained from \( pB1 \). To isolate the remaining cDNA sequence, a vegetative meristem (VM) cDNA library was screened [see Materials and methods]. Eight cDNAs were isolated from this screen. The sequence of these clones matched the composite cDNA sequence obtained from the ear cDNA clones described above. Sequence analysis of the cDNAs indicated that the 5' ends of all but one cDNA end within 50 bp of each other. The 5' ends of the VM cDNAs map near to a putative TATA box identified from sequence of a \( \lambda \) genomic clone, \( \lambda B73 \), isolated from a B73 inbred DNA library by hybridization with \( pB1 \) [Figs. 4 and 5]. The genomic organization of the \( rsl \) gene is shown in Figure 4. Sequence data from \( \lambda B73 \) were obtained for the entire gene except the internal portion of the third intron. Comparison of genomic and cDNA sequences confirmed the positions of all four introns. Consensus splice donor and acceptor sites were found in the genomic sequence of each exon/intron boundary. Two unspliced introns were found in the ear cDNA clones, however we have found no evidence of alternative mRNA processing. Unspliced in...

**Figure 4.** Restriction map of \( rsl \) B73 allele genomic clone \( \lambda B73 \). Open boxes show the location of exons 1-5, filled boxes show the homeo domain coding region. ATG and TGA indicate the locations of translational start and stop signals. The location of probes \( pB1 \) and \( p845P \) are shown as lines above \( \lambda B73 \). The cross-hatched triangle above \( \lambda B73 \) indicates the site of a \( Mutator \) \( 6/7 \) transposon insertion into the \( Rsl-1025 \) allele. Filled triangles indicate the approximate locations of \( Mutator\)-induced insertions/rearrangements associated with reversion of the \( Rsl-1-O \) phenotype. [B] BamHI; [E] EcoRI; [H] HindIII; [S] SstI; [X] XbaI.

**Figure 5.** The nucleotide sequence and predicted amino acid sequence of \( Rsl \). Numbers indicate amino acid residues. The putative TATA box and poly(A) addition signals are boxed. The underlined G indicates the 5' end of the longest cDNA isolated. Asterisks indicate the sites of poly(A) addition found in the cDNAs. The CAX/glutamine/histidine sequences are underlined. The positions of introns are shown by filled inverted triangles. A potential nuclear localization signal is double underlined. The homeo domain is outlined. The end of translation is indicated by a filled square. Numbers indicate amino acids. GenBank accession number L44133.
tron transcripts have been found in many plant cDNA sequences and may result from splicing failure (Nash and Walbot 1992). Figure 5 shows the nucleotide sequence of the rsl transcription unit and the deduced amino acid sequence of the RS1 protein. The longest open reading frame codes for a 38,878 protein that contains a homeo domain. Translations in all three reading frames revealed no transcription unit and the deduced amino acid sequence were not capable of detecting any transcripts in poly(A) + Northern blots of ear RNA, suggesting that the 5' end of the transcription unit lies near the end of the longest cDNA. An RNase protection experiment was performed with a 480-bp probe predicted to overlap the start site (p845P probe, Fig. 4). This experiment identified several start sites between the TATA sequence and the ATG (data not shown). These observations indicate that the open reading frame shown represents the entire RS1-coding sequence. Five polyadenylation sites were found at the 3' end of the rsl transcript (Fig. 5).

Several features of the predicted amino acid sequence are worth noting. In addition to the homeo domain, the protein sequence contains a nuclear localization signal (Raikhel 1992) in a basic region preceding the helix-turn-helix motif (Fig. 5, double underlined sequence). The amino-terminal 25 amino acids contain a glycine/serine-rich sequence reminiscent of the glycine loop domain found in structural and RNA binding proteins (Steinert et al. 1991). The amino-terminal region of the protein also contains glutamine/histidine-rich [singly underlined sequence in Fig. 5] and alanine/serine-rich repeated amino acid regions in close proximity to one another. The glutamine-rich sequence is coded for by a trinucleotide repeat, or microsatellite, of the structure CAX and the alanine-rich sequence is coded for by a CCN repeat. Glutamine-rich sequences can modulate transcriptional activity and occur in many transcription factors of various types including homeo domain proteins (Gerber et al. 1994).

The intron/exon structure of rsl is almost identical to kn1 and to knat 1 and 2 of Arabidopsis (see Fig. 6; Lincoln et al. 1994). The only difference between rsl and kn1 splice site placement is intron III, which occurs 27 nucleotides 5' of the kn1 site. knat 1 and 2 also show variation at the third intron/exon position (Lincoln et al. 1994). To examine the possibility of alternative splicing at this intron/exon position, sequence across the exon III/IV splice site of all eight vegetative meristem cDNAs was compared and found to have the same sequence obtained from an ear cDNA obtained through rapid amplification of cDNA ends (RACE; see Materials and methods), indicating that only one splice site is used for intron III.

A comparison between the predicted amino acid sequences of RS1 and KN1 proteins [Vollbrecht et al. 1991] is shown in Figure 6. The RS1 homeo domain is 87% identical (57/64 amino acids) to KN1. The third recognition helix is completely identical to KN1 and other knox homeo domains (Kerstetter et al. 1994), suggesting that these proteins may have the ability to bind the same DNA sequence. The conserved region termed the ELK sequence, adjacent to the amino terminus of the homeo domain, is 75% identical to KN1 (18/24). Sequence identity is 53% over the remaining 35 residues in the carboxyl terminus. The RS1 protein is characterized by two regions amino-terminal to the ELK sequence (Gehring et al. 1990). Filled triangles indicate the position of introns I-IV. The alignment was performed by use of the method of Wilbur and Lipman (1983) as implemented by MacVector Software (IBI-Kodak) with a hash value of 2 and using the pam250 matrix.

DNA sequence. The conserved region termed the ELK sequence, adjacent to the amino terminus of the homeo domain, is 75% identical to KN1 (18/24). Sequence identity is 53% over the remaining 35 residues in the carboxyl terminus. The RS1 protein is characterized by two regions amino-terminal to the ELK sequence. The region representing amino acids 85–228 displays high similarity to KN1 (53% identity) except in the area surrounding the third intron. The second region, from amino acid residue 1 to 84, shows no homology to KN1 except for the CAX repeat nucleotide sequence described above. In KN1 this sequence codes for a polyhistidine stretch. DNA homology searches and translations in all reading frames of the sequence covering the first 50 amino acids of RS1 showed no similarity to KN1.

The rsl transcript is ectopically expressed in leaves of rsl mutants

The wild-type pattern of rsl transcript accumulation was examined by RNA gel blot analysis. Poly(A)+ RNA
was prepared from immature organs and RNA gel blot filters were probed with an rs1 VM cDNA at high stringency. The highest level of transcript was detected in developing ears, whereas strong signals were also present in tassels and seedling mesocotyl (first seedling internode/node), and somewhat lower levels were present in roots (Fig. 7). Transcript was not detected in leaf blades or sheaths. Because Jackson and colleagues (Jackson et al. 1994) failed to detect rs1 in primary root apices rs1 expression is probably proximal to the root tip or is present at low levels in all cell types.

Three dominant mutant alleles of rs1 have been genetically characterized (Becraft and Freeling 1994). No detectable molecular weight difference was observed between the mature transcripts in the normal inbred line B73 compared to the mutants Rs1-O, Rs1-1025, or Rs1-Z4 (Fig. 8A). RNA was then isolated from the ligular region of wild-type leaves and leaves expressing each of the three mutant phenotypes. Because the Rs1-Z4 phenotype expresses in early leaves, whereas Rs1-O and Rs1-1025 express in later leaves, RNA was extracted from both early and late leaves of B73 inbred plants. rs1 transcripts were detected in leaves of all three mutants but not B73, even after over exposure of the autoradiograph, suggesting that ectopic expression of a normal rs1 transcript in leaves coincides with, and is the probable cause of, the mutant phenotype (Fig. 8B). Rehybridization of the same gel blot in Figure 8B with a kn1 gene probe did not show any increase in kn1 message in rs1 mutant tissue (data not shown). Therefore rs1 does not appear to activate kn1 transcription in the context of the leaf.

The cellular localization of rs1 transcript was investigated by in situ hybridization of digoxigenin-labeled RNA probes to tissue sections of vegetative shoot meristems from normal and mutant siblings (Fig. 9). In transverse sections of wild-type meristems, rs1 expression was visualized as a ring of signal occurring at each site of leaf insertion into the central axis of the shoot (Fig. 9A, B, C). In longitudinal sections, each ring of expression appears as either two points of signal comprising 5-10 cells each on opposite flanks of the apex, or as a lateral stripe of signal, depending on the plane of bisection (Fig. 9A, C). The earliest appearance of rs1 expression occurs in the apical meristem, coincident or preceding plastochron 0, before any visible signs of leaf initiation (Fig. 9A). Diffuse signal can be observed in more apical re-
regions of the meristem and may represent the initiation of the rs1 pattern.

rs1 expression in the shoot axis is localized to the abaxial side of the leaf insertion point and is not present over the base of the leaf insertion point (arrows in Fig. 9A,D). In transverse section rs1 expression is not seen at the site of P2 insertion but can be seen opposite the P2 leaf and corresponds to expression subtending the P1 leaf on the other side of the meristem (Fig. 9G, arrow). rs1 expression is localized at the insertion point of older leaves and is most intense at the site of axillary meristem initiation, opposite to the leaf it subtends (Fig. 9D). Expression can also be seen in some vascular elements in the stem. The early expression of rs1 suggests it may be

Figure 9. (See facing page for legend.)
In later stages of inflorescence development, meristem shows that normal siblings shows that mordia and also subtends lateral structures such as nonexpressing, semicircular regions that correspond to not show ectopic expression and appears outlined by defines the phytomer (Galinat 1994) or leaf/node boundaries. Expression in any part of the leaf proper was never observed in normal plants.

rs1 expression persists in the transition from vegetative to inflorescence meristem, however the pattern of expression is more extensive and appears to outline several lateral regions on the sides of the meristem (Fig. 9D). In later stages of inflorescence development rs1 expression can be seen at the site of each future floral primordium. (Fig. 9E). A tangential section of the inflorescence meristem shows that rs1 expression outlines a series of nonexpressing, semicircular regions that correspond to the sites of lateral floral meristem initiation (Fig. 9F). Later in floral development rs1 is expressed in floral primordia and also subtends lateral structures such as glumes in floral meristems (Fig. 9E arrows and data not shown). Thus, rs1 expression precedes initiation of and subtends or surrounds the sites of all lateral primordia derived from the shoot apex. Control experiments with rs1 sense-RNA probes did not produce any signal.

Comparison of sections of Rs1-O shoot apices with normal siblings shows that Rs1 is ectopically expressed in leaves as young as plastochron 1 (P1) (Fig. 9, cf. A with H; G with J, K, and L). In panel G no signal is apparent at the site of P2 leaf insertion, whereas the Rs1-O apex (Fig 9H,J) shows more extensive and uniform Rs1-O expression than the wild-type. Interestingly, the midvein does not show ectopic expression and appears outlined by Rs1 ectopic expression in neighboring cells (Fig. 9G). Ectopic expression can be seen in regions above the site of leaf insertion in panels K and L and also in the lower regions of a P1 leaf (Fig. 9H,L). The region of ectopic expression includes the region of ligule initiation suggesting that ectopically expressed Rs1 interferes with normal ligule development at a stage that coincides with ligule initiation (Sylvestre et al. 1990). Ectopic expression in mutant leaves appears to occur in most cell types, with a higher concentration surrounding the developing vasculature in P2 leaves. Ectopic expression can also be seen in P3 leaves and is more uniform around the midvein region of the developing leaf (Figure 9I). In longitudinal section of mutants, the Rs1 expression domain in the meristem is broader than normal and distinct rings of expression at the site of leaf insertion into the shoot are not observed in all apices (Fig. 9, cf. H with A). Rs1 is expressed in a larger region over the site of leaf insertion and lower levels of ectopic expression can be seen in all leaves, including P1, compared to normal apices (Fig. 9, cf. A with H).

Ectopic Rs1 transcript has also been localized to tissues where there is no obvious morphological difference between mutant and wild-type, including the vasculature of sheath, blade tissue, and ground tissue of the shoot (Fig. 9H,I). These results indicate that some factor other than transcriptional regulation of Rs1 may be involved in the specificity of the Rs1 mutant phenotype for the ligular region of the leaf.

**Figure 9.** In situ hybridizations of digoxigenin labeled rs1 probe to sectioned normal and Rs1-O/+ heterozygous siblings. (A) Median, longitudinal section of wild-type, 2-week-old maize shoot apex. Note the stripes of rs1 expression in the shoot apical meristem and the localized foci of expression at the base of insertion of each leaf [small arrows]. (B) In transverse section each stripe appears as a circle of rs1 expression. The large arrow in A shows the position of the transverse section in B. (C) Longitudinal section of a normal shoot apex showing bisection of the ring of expression subtending leaf insertion. (D) Longitudinal section of a 4-week-old normal, early inflorescence meristem. Rs1 transcripts are localized as several paired, semicircular shaped structures in the region of developing tassel branches and floral meristems at the base of the tassel primordium. Rs1 expression at the base of leaf insertion can be observed for several plastochrons especially in the region of axillary bud development [small arrows]. (E) Later stage tassel primordium showing rs1 expression at each spikelet pair primordium [arrows]. (F) A section tangential to the meristem in E showing a ladder-like rs1 expression pattern [the lines show where the region shown can be localized approximately in E]. (G) Transverse section at the site of P2 leaf insertion into the stem. The yellow outline shows the border between the P3 leaf and the site of P2 leaf insertion. The light blue signal on the opposite side of the P2 leaf indicated by an arrow shows rs1 expression at the base of the P1 leaf. (H–L) Ectopic expression in Rs1 mutants. (H–L) Serial sections, 40 μm apart, from the P2 leaf insertion toward the shoot apex. The yellow outlines show the extent of developing P2 leaf primordia. Ectopic expression can be seen in all P2 leaves and in the P1 primordia (L, arrow). Expression is also increased over the site of leaf insertion in young leaves that corresponds to the region of ligule initiation and development. The large arrow in H shows the approximate position of the transverse section of the Rs1-O/+ shoot in J. (M) Meristem; (IM) inflorescence meristem. P1, 2, and 3 refer to Plastochron 1, 2, and 3 leaves. Bars, 100 μm.

**Figure 10.** RNA gel blot analysis of Rs1-Or90 and Rs1-Or122 revertant homozygotes. (A) RNA gel blot of 25 μg of total RNA isolated from ear primordia of Rs1-O r90 and Rs1-O r122 homozygotes and wild-type homozygote siblings, hybridized with Rs1 cDNA pVM4.1. (B) Ethidium bromine-stained 25S ribosomal RNA corresponding to the above lanes.
and r312 heterozygous, phenotypic revertants were analyzed for rs1 expression and potential loss of function phenotypes. Forty seeds from each self-pollinated revertant heterozygote were planted. DNA gel blot analysis with the DNA polymorphisms shown in Figure 3 was performed on all viable individuals to determine their genotype. Both r90 and r122 produced a normal segregation ratio of homozygous wild-type, mutant and heterozygous mutant genotypes. RNA gel blot analysis of immature ear tissue was performed on homozygous Rs1-Or90 and r122 plants to determine if these alleles still produced rs1 message of the appropriate size and quantity. Figure 10 shows that both alleles produce transcripts of normal size and abundance, indicating that the Rs1-Or90 and r122 alleles represent reversion from dominant to normal RNA expression patterns. Self-fertilized progeny were screened at the seed, seedling, and mature stages of development. No phenotypes linked to homozygosity for r90 or r122 Rs1-O polymorphisms were observed.

Analysis of Rs1-Or312/+ self-fertilized progeny identified an embryo lethal phenotype segregating at the frequency of 1:3. DNA gel blot analysis of the viable progeny from a self fertilized heterozygote identified 9 homozygous wild-types, 21 Rs1-Or312 heterozygotes [Rs1-Or312/+], 1 homozygous Rs1-Or312 revertant and 10, nongerminating, embryo lethal individuals. These results indicate that the embryo lethal phenotype is closely linked to the Rs1-Or312 polymorphism. Further studies of this revertant are being conducted to determine if the embryo lethal phenotype is caused by loss of rs1 function.

Discussion

We describe the isolation of the maize homeo box gene rough sheath1 based on genetic and molecular similarity to the knotted1 homeo box gene. rough sheath1 and knotted1 represent two members of a family of highly related maize homeo box genes for which dominant mutations in many result in leaf developmental abnormalities (Freeling 1992). Following the identification of the kn1 homeo box numerous plant homeo box genes have been identified by use of both molecular and genetic approaches (Ruberti et al. 1991; Bellmann and Werr 1992, Mattsson et al. 1992; Schena and Davis 1992; Matsuoka et al. 1993; Schindler et al. 1993; Korfhage et al. 1994; Lincoln et al. 1994; Rerie et al. 1994; Quaedvlieg et al. 1995). A number of distinct classes of homeo box genes have emerged from these studies including unique types such as the HD-Zip proteins, which have a leucine zipper motif in combination with the homeo box (Schena and Davis 1992). The function of most plant homeo box genes is unknown, however several have been identified on the basis of their function in light and defense gene regulation (Schindler et al. 1993; Korfhage et al. 1994; Quaedvlieg et al. 1995). The glabra2 (gl2) gene represents a distinct class of homeo box genes that functions in trichome development. Recessive mutations of gl2 result in defective trichome expansion, suggesting that gl2 functions in cell-fate specification [Rerie et al. 1994]. Dominant mutations of the maize Kn1 and Rs1 genes [Becraft and Freeling 1994; Sinha and Hake 1994] and the barley Hooded gene [Müller et al. 1995] also result in cell fate alterations and homeotic transformations, suggesting that the function of the homeo box motif as a regulator of cell fate has been conserved throughout the evolution of multicellular organisms.

The Rs1 phenotype for three different alleles is due to ectopic expression of Rs1 transcripts in leaf primordia (Figs. 8B and 9). The mechanism by which this occurs is not known, however the Rs1-1025 mutation is caused by a Mutator transposon insertion into the third intron of the gene. Studies of Kn1 mutations have shown that many are due to transposon insertion into a relatively small region of the third intron [Greene et al. 1994]. In addition, the barley Hooded mutation, which results from over expression of a kn1 like gene, is apparently caused by a small duplication in the third intron [Müller et al. 1995]. One possibility is that mutation of a regulatory element contained in the third intron causes ectopic/over expression in leaf organs. However, other mechanisms are possible because the kn1-0 allele is caused by tandem duplication of the kn1 gene (Veit et al. 1992) and transgenic over expression of kn1 and rice OSH1 homeo box genes results in a knotted-like phenotype (R. Williams, S. Hake, and P. Lemaux, pers. comm; Matsuoka et al. 1993).

The cellular expression pattern of rs1 as well as several other KNOX class I genes shows a very interesting repetitive pattern that is related to the segmental structure of the grass shoot (Fig. 9; Jackson et al. 1994). A ring of rs1 expression is observed at each leaf insertion point in the apical meristem. rs1 expression appears in the apex before any visible sign of leaf initiation, suggesting that rs1 may be involved in, or respond to, an early pattern forming event that defines the segmental unit of the shoot axis or leaf boundaries. The segmental pattern continues in the inflorescence and floral meristems, where rs1 expression appears to isolate many similarly sized groups of cells which represent precursor cells of floral meristems (Fig. 9E,F). The consistent expression of rs1 at all sites of lateral organ initiation suggests that one function of rs1 may be to partition groups of cells into developmental units. Clonal analysis studies of vegetative development in maize have shown that each newly initiated leaf represents the coordinated development of both the leaf and associated node, internode and axillary bud (Poethig 1984; Poethig and Szymbowiak 1995). These structures are all descended from a group of ~100-200 founder cells in the meristem. Interestingly, the expression patterns of rs1 and kn1 appear to subend and outline this region of cells in the meristem, respectively, suggesting that knox class I genes may have a direct role in partitioning cells in the meristem. The expression domains of rs1 and kn1 are distinct yet contain substantial overlap, particularly in meristems (Fig. 9; Jackson et al. 1994). However, neither rs1 or kn1 appear to regulate each other in the context of ectopic expression in the leaf.
[This study and Jackson et al. 1994]. It will be of great interest to determine if genes such as \textit{rs1} have a molecular role in the repeated ontogeny of plant organs.

Both \textit{Rs1} and \textit{Kn1} mutants impose sheath-like characteristics on blade regions of the leaf, yet unexpectedly, neither gene is normally expressed in the sheath. In \textit{Drosophila}, segment identity is determined by the action of homeotic genes. Loss-of-function mutations in these genes cause homeotic transformations of the segments where they are normally expressed and whose identity they control (for review, see McGinnis and Krumlauf 1992). Ectopic expression usually confers characteristics of the normal site of expression on the ectopic site. Homeotic genes that control floral organ identity in \textit{Arabidopsis} and \textit{Antirrhinum} act similarly [Bowman et al. 1989; Coen and Meyerowitz 1991; Drews et al. 1991; Mizukami and Ma 1992; Bradley et al. 1993]. \textit{rs1} and \textit{kn1} are both expressed in the stem, which has many of the characteristics of the mutant leaf tissue; it is thick and stiff, and would normally continue growing after the blade and sheath have stopped. Genetic, morphological, and histological analyses, however, have consistently identified the mutant tissue as sheath-like [Freeling and Hake 1985; Becraft and Freeling 1989, 1994; Hake 1992, Sinha and Hake 1994]. It is possible that the phenotype is due to two separable components, a cell differentiation phenotype [sheath like] and a cell division phenotype (stem). Mixed segmental phenotypes have been observed in several UBX/ANTP chimeric protein over expression studies in \textit{Drosophila} [Chan and Mann 1993].

There are several possible explanations for how ectopic expression of \textit{Rs1} and \textit{Kn1} could change the fate of blade cells to sheath. One possibility is that a gene or genes that determine sheath identity are activated by \textit{rs1} and \textit{kn1} in leaf cells. Other possibilities relate to the hypothesis that the sheath represents the developmental ground state of the leaf onto which genetic programs are superimposed to determine blade [Freeling 1992]. \textit{Rs1} and \textit{Kn1} could repress genes that specify blade identity, thus leaving the affected cells in the ground state identity of sheath. Alternatively they may compete with homeo domain proteins required for blade fate, thereby retarding the acquisition of blade identity as suggested by Freeling [1992]. The last model is that \textit{Rs1} and \textit{Kn1} promote an indeterminate state of leaf cells that prevents them from acquiring a blade fate [Hake 1992; Sinha et al. 1993].

An interesting aspect of the dominant homeo box mutants in maize is the phenotypic specificity for distinct regions of the leaf displayed by each mutant. Multiple alleles of \textit{Kn1} affect primarily lateral veins of the blade, whereas \textit{Rs1} alleles affect the whole ligular region and base of the blade [Freeling and Hake 1985; Freeling 1992; Hake 1992; Becraft and Freeling 1994]. \textit{Lg3} and \textit{Lg4} mutants have other distinct specificities [Fowler 1994]. Ectopic expression of \textit{kn1} in the veins predicts the placement of mutant cells in \textit{Kotted} leaves [Smith et al. 1992], however, expression patterns do not fully explain the tissue-specific mutant phenotype of \textit{Rs1-O}. The region of ectopic expression of \textit{rs1} transcript appears larger than the phenotypically affected area [Fig. 9H–L] suggesting that only target regions of the leaf may be competent to respond to ectopic expression of particular genes. Examples of cell-type-specific regulation of target genes by homeo domain proteins are reviewed in Hayashi and Scott [1990]. Competency could be determined by region-specific factors or by some temporal component of leaf development [Freeling 1992].

Mutagenesis of dominant mutants commonly produces recessive loss-of-function alleles [Lifschitz and Falk 1969; Lifschitz and Green 1979]. In this study, 15 of 19 \textit{Mutator} transposon-induced revertants showed recurrent \textit{Rs1} mutant phenotypes. This indicates that the original plants selected from the screen were partial revertants, or that the reversion was suppressed. No evidence of a second-site suppressor has been found to date. Analysis of four full revertants showed one to result from a deletion of unknown size and three to contain DNA rearrangements consistent with \textit{Mutator} transposon insertions in different regions of the gene [Fig. 4]. Segregation analysis of the \textit{Rs1-Or90}, \textit{r122}, and \textit{r312} alleles showed that only \textit{Rs1-Or312} was closely linked to a recessive embryo lethal phenotype. The \textit{Rs1-Or312} allele may be \textit{Mutator} suppressible and require \textit{Mutator} activity to effect the mutant phenotype [Barkan and Martinssen 1991]. The \textit{Rs1-Or90} and \textit{r122} alleles still express \textit{rs1} mRNA and therefore represent reversion to a wild-type gene expression pattern [Fig. 10]. This result is consistent with the location of DNA insertions in noncoding regions of the gene and suggests that these insertions eliminate ectopic expression while maintaining normal expression patterns [Fig. 4].

Genetic studies have shown that the \textit{Rs1-O} mutant acts cell nonautonomously in the leaf, that it shows no effect of mutant allele dosage and that many of the phenotypic effects are similar to effects caused by auxins [Becraft and Freeling 1994]. This may be analogous to the situation in \textit{Drosophila} where the \textit{Ultra} and \textit{abdominalA} genes nonautonomously induce the expression of \textit{labial} in cells adjacent to their expression dom-ains [Immergluck et al. 1990; Reuter et al. 1990]. This nonautonomous regulation requires the action of \textit{decapentaplegic}, which encodes a \textit{TGF-β} like protein [Padgett et al. 1987] that is the apparent inducing signal. \textit{Rs1-O} may similarly be regulating the production of a growth factor [e.g. auxin] that gives a nonautonomous phenotype and could affect cells differently depending on their competencies.

Materials and methods

\textit{Nucleic acid analyses}

DNA for genomic DNA gel blot analysis was extracted from 0.7 grams of leaf tissue as described by Chen and Dellaporta [1994]. The DNA was resuspended in 300 μl of TE, and 15 μl was used per restriction enzyme digest. The DNA was digested with \textit{BamHI}, \textit{EcoRI}, \textit{ HindIII} [New England Biolabs], or \textit{SstI} [GIBCO BRL] using the manufacturer’s buffers. The restricted DNA was electrophoresed through 0.8% agarose gels and transferred to
Schneebberger et al.

Duralon membranes [Stratagene] according to the manufacturer's specifications. The DNA was immobilized onto the membrane by UV cross-linking in a Stratalinker [Stratagene]. RNA was prepared from tissues as described previously (Kloeckener-Gruissem et al. 1992) and poly[A]* selected with oligo(dT) cellulose columns (Sambrook et al. 1989). The RNA for Northern analysis was subjected to electrophoresis through formaldehyde gels of 1% agarose, blotted onto Duralon membranes [Stratagene] and immobilized by UV cross-linking in a Stratalinker [Stratagene].

Radiolabeled probes were made by incorporation of [32p]dCTP [NEA] with a Prime It random primer kit [Stratagene]. The 247-bp knl homeo box fragment was generated from the cloned cDNA (Vollbrecht et al. 1991) though PCR amplification of the fragment corresponding to bases 924 to 1160. The fragment was purified in a 1% SeaPlaque GTG [FMC] agarose gel.

Low-stringency hybridizations for genomic DNA gel blots and plaque lift screening were done overnight at 65°C in 9× SSC and washed twice for 45 min in 1× SSC at 55°C. High-stringency hybridizations were done overnight at 65°C in 6× SSC and washed in 0.2× SSC at 65°C twice for 1 hr. RNA hybridizations were done under the same high-stringency conditions except the washes were done in 0.1× SSC.

Genomic cloning

SstI-digested DNA from a Rs1-O plant was enriched for 2-kb fragments by size fractionation through a sucrose gradient, and a library constructed in SstI-digested Z Zap [Stratagene]. The library was packaged with Gigapack packaging extract [Stratagene] and plated onto BB4 cells supplied with the vector. Plaques (1× 10⁶) were screened by probing of nitrocellulose filter lifts at low stringency with the knl homeobox probe. Hybridizing plaques were picked, used to inoculate 2-ml cell cultures and phage DNA was prepared from the lysate [Sambrook et al. 1989]. A Bluescript plasmid containing the insert was recovered by use of the Zap in vivo excision method (Stratagene).

The 2-kb Rs1-O SstI fragment was used as a probe to isolate an ~18-kb EcoRI fragment from the commercial inbred line B73 (A8873 in Fig. 4) and a 9-kb EcoRI fragment from the Rs1-1025 mutant. DNA from each line was digested with EcoRI and enriched for the respective fragment sizes by sucrose gradient fractionation. Libraries were constructed by ligation of the genomic fragments into EcoRI digested ADASH [Stratagene]. The library was packaged with Gigapack packaging extract [Stratagene] and plated onto SRBP2 cells. Plaque lifts were screened at high stringency with the rs1 2-kb SstI fragment.

cDNA isolation

Four cDNA clones, EC1, EC6, EC20, and EC21 were kindly provided by Bruce Veit as knl homologous isolates from an ear cDNA library. EC1 was the longest cDNA clone covering 800 bp of the predicted 1.6-kb message length and contained a complete unspliced intron IV and 200 bp of unspliced intron III. Rapid amplification of cDNA ends (RACE; kit purchased from BRL) with a gene-specific primer located 33 bp 3' of the third intron (5'-accattcgtgtcgatctc-3'), based on EC1 sequence, produced a 256-bp cDNA, pRs5.20, which spans the exon III/IV splice site. The structure and sequence derived above was verified by isolation and sequence analysis of seven near full-length cDNA clones from a vegetative meristem cDNA library [provided by B. Veit] with pRs5.20 as a probe. Consensus 5' and 3' splice recognition sites were found in the genomic sequence for introns I, II, III, and IV (Goodall et al. 1991). Vegetative meristem cDNA clone pVM4.1 is used as the hybridization probe in Figures 7 and 10.

DNA sequencing

Double-stranded plasmid templates were prepared and sequenced on both strands with a Sequenase 2.0 kit [U.S. Biochemical] according to the manufacturer's instructions. Terminated fragments were labeled by incorporation of [35S]ATP [NEA], resolved on 6% acrylamide gels, and autoradiographed on Kodak XAR5 film.

In situ hybridizations

Tissue samples were fixed in FAA [Formalin/acetic acid/ethanol] and embedded in paraffin as described by [Jackson 1991]. Sections (10-μm) were affixed to Probe-On Plus microscope slides [Fisher] and given the prehybridization treatments described previously [Jackson 1991] except the pronase step was replaced with proteinase K for 30 min at 37°C. The probes were prepared from a ear rs1 cDNA from which the sequence 3' from the SpRL site at nucleotide 1613 was deleted to remove the poly(A) tail. The probe extends from the beginning of the third exon to nucleotide 1613. RNA transcriptions were performed from the Bluescript SK+ plasmid [Stratagene] containing the insert by use of T3 RNA polymerase to create the sense probe and T7 RNA polymerase to make antisense. Both polymerases were purchased from New England Biolabs and the manufacturer's buffers were used. Digoxigenin-labeled UTP (Boehringer Mannheim) was incorporated into each transcript at a ratio of 1:1 with UTP. The probes were hydrolyzed to an average length of ~150 bases and hybridizations carried out under the conditions described [Jackson 1991] in a Microprobe Regent [Fisher]. Slides were then washed in 0.2× SSC at 50°C twice for 1 hr. The slides were rinsed twice for 5 min in NTE buffer (0.5 M NaCl, 10 mM Tris at pH 7.5, 1 mM EDTA) and then treated with 20 mg/ml RNase A in NTE at 37°C. They were then rinsed twice for 5 min in NTE and twice for 1 hr in 0.2× SSC at room temperature. The slides were stored overnight at 4°C in PBS (130 mM NaCl, 10 mM NaHPO₄, at pH 7.0). Immunological detection was done by use of a Boehringer Mannheim digoxigenin-nucleic acid detection kit as described by Coen et al. [1990]. The alkaline phosphatase-detected slides were dehydrated in an ethanol series, washed in Histoclear [National Diagnostics] and mounted with Merkogloss [EM Science]. Competition experiments with an unlabeled knl probe showed no change in signal localization or intensity, indicating that the probe is specific for rs1 transcripts.

Acknowledgments

The authors thank Rosanna Lachmansingh for technical assistance, Bruce Veit [Massey University, New Zealand] for providing the ear rs1 cDNA clones and the vegetative meristem library, David Jackson for advice with in situ hybridizations, and Randall Tyres for assistance with sequence analysis. We are also grateful to Steve Ruzin and the NSF Center for Plant Developmental Biology for providing facilities and technical advice. We also thank Gary Muchlbaier and Mark Mooney for comments on the manuscript. This research was supported by a grant from National Institutes of Health to M.F. and NIH fellowship F32GM14578-02 to R.G.S.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby

2302 GENES & DEVELOPMENT
marked “advertisement” in accordance with 18 USC section 1734 solely to indicate this fact.

References

Barkan, A. and R.A. Martienssen. 1991. Inactivation of maize transposon Mu suppresses a mutant phenotype by activating an outward reading promoter near the end of Mut. Proc. Natl. Acad. Sci. 88: 3502–3506.

Becraft, P.W. and M. Freeling. 1989. Use of the scanning electron microscope to ascribe leaf regional identities even when normal anatomy is disrupted. Maize Genet. Coop. Newsbl. 63: 37.

——. 1994. Genetic analysis of rough sheath1 developmental mutants of maize. Genetics 136: 295-311.

Bellmann, R. and W. Werr. 1992. Zmhoxla, the product of a 65 bp feedback control element. EMBO J. 11: 3367–3374.

Bradley, D., R. Carpenter, H. Sommer, N. Hartley, and E. Coen. 1993. Complementary floral homeotic phenotypes result from opposite orientations of a transposon at the plena locus of Antirrhinum. Cell 72: 85–95.

Bowman, J.L., D.R. Smyth, and E.M. Meyerowitz. 1989. Genes directing flower development in Arabidopsis. Plant Cell 1: 37–52.

Chan, S.-K. and R.S. Mann. 1993. The segment identity functions of Ultrathorax are contained within its homeo domain and carboxy-terminal sequences. Genes & Dev. 7: 796–811.

Chandler V.L. and K.J. Hardeman. 1992. The Mu elements of Zea mays. Adv. Genet. 30: 77–122.

Chen, J. and S. Dellaporta. 1994. Urea based plant DNA mini-preps. In The maize handbook (ed. M. Freeling and V. Wallbot), pp. 526–527. Springer-Verlag, New York.

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

Coen, E.S., J.M. Romero, S. Doyle, R. Elliot, G. Murphy, and R. Carpenter. 1990. florecula: A homeotic gene required for flower development in Antirrhinum majus. Cell 63: 1311–1322.

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

Fowler, J.E. 1994. “Genetic and molecular analysis of dominant Liguleless mutations in maize.” Ph.D. thesis, University of California, Berkeley, Berkeley, CA.

Freeing, M. 1992. A conceptual framework for maize leaf development. Dev. Biol. 153: 44–58.

Freeing, M. and S. Hake. 1985. Developmental genetics of mutants that specify knotted leaves in maize. Genetics 111: 617–634.

Galina, W.C. 1994. The patterns of plant structures in maize. In The maize handbook (ed. M. Freeing and V. Wallbot), pp. 61–65. Springer-Verlag, New York.

Gehring, W.J., M. Müller, M. Affolter, A. Percival-Smith, M. Billeter, Y. Qian, G. Otting, and K. Wüthrich. 1990. The structure of the homeo domain and its functional implications. Trends Genet. 6: 322–329.

Gehring, W.J., Y.Q. Qian, M. Billeter, K. Furukubo-Tokunaga, A.S. Shier, D. Resendez-Perez, M. Affolter, G. Otting and K. Wüthrich. 1994. Homeodomain-DNA recognition. Cell 78: 211–223.

Galinat, W.C. 1994. The patterns of plant structures in maize. Adv. Genet. 30: 77–122.

Greene B., R. Walko, and S. Hake. 1994. Mutator insertions in an intron of the maize knotted1 gene result in dominant suppressible mutations. Genetics 138: 1275–1285.

Goodall, G.J., T. Kiss, and W. Filipowicz. 1991. Nuclear RNA splicing and small nuclear RNAs and their genes in higher plants. In Oxford surveys of plant molecular and cell biology (ed. B.J. Mifflin), vol. 7, pp. 255–296. Oxford University Press, Oxford, UK.

Hake, S. 1992. Unraveling the knots in plant development. Trends Genet. 8: 109–114.

Hake, S. and M. Freeling. 1986. Analysis of genetic mosaics shows that the extra epidermal cell divisions in Knotted mutant maize plants are induced by adjacent mesophyll cells. Nature 320: 621–623.

Hake, S., E. Vollbrecht, and M. Freeing. 1989. Cloning Knotted, the dominant morphological mutant of maize using Ds2 as a transposon tag. EMBO J. 8: 15–22.

Hayashi, S. and M.P. Scott. 1990. What determines the specificity of action of Drosophila homeo domain proteins? Cell 62: 883–894.

Immergluck, K., P.A. Lawrence, and M. Bienz. 1990. Induction across germ layers in Drosophila mediated by a genetic cascade. Cell 62: 261–268.

Jackson, D. 1991. In situ hybridisation in plants. In Molecular plant pathology: A practical approach (ed. D.J. Bowles, S.J. Gurr, and M. McPherson), pp. 163–174. Oxford University Press, Oxford, England.

Jackson, D., B. Veit, and S. Hake. 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.

Kenyon, C. 1994. If birds can fly, why can’t we? Homeotic genes and evolution. Cell 78: 175–180.

Kerstetter, R., E. Vollbrecht, B. Lowe, B. Veit, J. Yamaguchi, and S. Hake. 1994. Sequence analysis and expression patterns divide the maize knotted1-like homeobox genes into two classes. Plant Cell 6: 1877–1897.

Kloeckener-Gruissem, B., J.M. Vogel, and M. Freeing. 1992. The TATA box promoter region of maize Adh1 affects organ-specific expression. EMBO J. 11: 157–166.

Korfhage, U., G.F. Trezzini, I. Meier, K. Halbrock, and I.E. Lifschitz. 1979. Genetic identification of killer-phenotype mutants. II. The abnormal growth conditioned by the killer mutant. Mol. Gen. Genet. 136: 295-311.

Laughon, A. and M.P. Scott. 1984. Sequence of a Drosophila segmentation gene: Protein structure and homology with DNA binding proteins. Nature 310: 25–31.

Lawrence P.A and G. Morata. 1994. Homeobox genes: Their function in Drosophila segmentation and pattern formation. Cell 78: 181–191.

Levine, M. and T. Hoey. 1988. Homeobox proteins as sequence specific transcription factors. Cell 55: 537–540.

Lifschez, E. and R. Falk. 1969. A genetic analysis of the killer-prune ([k-pn]) locus of Drosophila melanogaster. Genetics 62: 353–358.

Lifschez, E. and M.M. Green. 1979. Genetic identification of dominant overproducing mutations: The Beadex gene. Mol. Gen. Genet. 171: 153–159.

Lincoln, C., J. Long, J. Yamaguchi, K. Serikawa, and S. Hake. 1994. A knotted1-like homeobox gene in Arabidopsis is ex-
pressed in the vegetative meristem and dramatically alters leaf morphology when overexpressed in transgenic plants. *Plant Cell* 6: 1859–1876.

Matsuoka, M., H. Ichikawa, A. Saito, Y. Tada, T. Fujimura, and Y. Kano-Murakami. 1993. Expression of a rice homeobox gene causes altered morphology of transgenic plants. *Plant Cell* 5: 1039–1048.

Mattsson, J., E. Soderman, M. Svenson, C. Borkird, and P. Engstrom. 1992. A new homeobox-leucine zipper gene from *Arabidopsis thaliana*. *Plant Mol. Biol.* 18: 1019–1022.

McGinnis, W. and R. Krumlauf. 1992. Homeobox genes and axial patterning. *Cell* 68: 283–302.

Mizukami, Y. and H. Ma. 1992. Ectopic expression of the floral homeotic gene AGAMOUS in transgenic Arabidopsis plants alters floral organ identity. *Cell* 71: 119–131.

Müller, K.J., N. Romano, O. Gerstner, F. Garcia-Maroto, C. Pozzi, F. Salamini, and W. Rohde. 1995. The barley Hooded mutation caused by a duplication in a homeobox gene intron. *Nature* 374: 727–730.

Nash, J. and V. Walbot. 1992. Bronze-2 gene expression and intron splicing patterns in cells and tissues of *Zea mays* L. *Plant Physiol.* 100: 464–471.

Padgett, R.W., R.D. St. Johnston, and W.M. Gelbart. 1987. A transcript from a *Drosophila* pattern gene predicts a protein homologous to the transforming growth factor-β family. *Nature* 325: 81–84.

Poethig, R.S. 1984. Cellular parameters of leaf morphogenesis in maize and tobacco. In *Contemporary problems of plant anatomy* (ed. R.A. White and W.C. Dickson), pp. 235–259. Academic Press, New York.

Poethig, R.S. and E.J. Szymbowiak. 1995. Clonal analysis of leaf development in maize. *Maydica* 40: 67–76.

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

Raikhel, N.V. 1992. Nuclear targeting in plants. *Plant Physiol.* 100: 1627–1632.

Rete, W.G., K.A. Feldman, and M.D. Marks. 1994. The GLABRA2 gene encodes a homeo domain protein required for normal trichome development. *Genes & Dev.* 8: 1388–1399.

Reuter, R., G.E.F. Panganiban, F.M. Hoffmann, and M.P. Scott. 1990. Homeotic genes regulate the spatial expression of putative growth factors in the visceral mesoderm of *Drosophila* embryos. *Development* 110: 1031–1041.

Ruberti, I., G. Sessa, S. Lucchetti, and G. Morelli. 1991. A novel class of plant proteins containing a homeo domain with a closely linked leucine zipper motif. *EMBO J.* 10: 1787–1791.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: A laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Schenk, M. and R.W. Davis. 1992. HD-Zip proteins: Members of an Arabidopsis homeodomain protein superfamily. *Proc. Natl. Acad. Sci.* 89: 3894–3898.

Schlünder, U., H. Beckmann, and A.R. Cashmore. 1993. HAT3.1, a novel Arabidopsis homeodomain protein containing a conserved cysteine-rich region. *Plant J.* 4: 137–150.

Scott, M.P., J.W. Tamkun, and G.W. Hartzell, III. 1989. The structure and function of the homeo domain. *Biochim. Biophys. Acta* 989: 25–48.

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

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

Steinert, P.M., J.W. Mack, B.P. Korge, S.Q. Gan, S.R. Haynes, and A.C. Steven. 1991. Glycine loops in proteins: Their occurrence in certain intermediate filament chains, loricins and single strand RNA binding proteins. *Int. J. Biol. Macromolecules* 13: 130–139.

Sylvestre, A.W., W.Z. Cande, and M. Freeling. 1990. Division and differentiation during normal and *liguleless-1* maize leaf development. *Development* 110: 985–1000.

Veit, B., E. Vollbrecht, J. Matern, and S. Hake. 1990. A tandem duplication causes the *Kn1-O* allele of *Knotted*, a dominant morphological mutant of maize. *Genetics* 125: 623–631.

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

Wilbur, W.J. and D.J. Lipman. 1983. Rapid similarity searches of nucleic acid and protein databanks. *Proc. Natl. Acad. Sci.* 80: 726–730.
Ectopic expression of the knox homeo box gene rough sheath1 alters cell fate in the maize leaf.

R G Schneeberger, P W Becraft, S Hake, et al.

*Genes Dev.* 1995, 9:
Access the most recent version at doi:10.1101/gad.9.18.2292