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

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***Rough sheath1* (*Rs1*) is a dominant mutation that alters cell fate and causes unregulated cell division and expansion in the maize leaf. A *knox* (*Kn1* like-homeo box gene) sequence closely linked to the *Rs1-O* mutation was cloned and shown by transposon mutagenesis to encode the *rs1* 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. *rs1* 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 *rs1* expression subtend leaf insertion sites in the shoot, and lateral organ primordia in inflorescence and floral meristems. The timing and position of *rs1* expression in meristems suggests a possible role for *rs1* in patterning the placement of lateral organs along the axis of the shoot. In contrast to wild type, *rs1* is expressed in early leaf primordia of *Rs1* mutant plants, suggesting that ectopic expression causes the mutant phenotype. Ectopic expression in *Rs1-O* plants suggests the lingular region is more competent to respond to *rs1* than blade or sheath tissues.**

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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 (Bercraft 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 (Bercraft 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; Bercraft 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

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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.

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 *Bam*HI, *Eco*RI, *Hind*III, or *Sst*I, 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 *Sst*I 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 *Sst*I fragment, indicating that this genomic fragment was genetically linked to *rs1* within 1.2 map units.

A subgenomic *Sst*I 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 *Sst*I-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 homozygous 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 heterozygous *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 2. Cosegregation of a *kn1*-like homeo box sequence with the *Rs1-O* mutant phenotype. (A) A DNA gel blot of *Sst*I-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 *Sst*I-digested DNA samples from a family segregating for *Rs1-O*, probed at high stringency with the 2-kb *Sst*I 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.

DNA gel blotting (Fig. 3). DNA gel blots prepared with either *Hind*III- (lanes 1–9) or *Sst*I- (lanes 10–15) digested DNA were probed with pB1 or p845P (a 480-bp *Pst*I 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

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. Schneeberger, 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

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 *Hind*III or *Sst*I and the blots were probed with either pB1 or p845P (Fig. 4). (Lanes 1–4) *Hind*III-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 *Hind*III and in lanes 10–15 is digested with *Sst*I. 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.

Figure 4. Restriction map of *rs1* B73 allele genomic clone λ8B73. 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 λ8B73. The cross-hatched triangle above λ8B73 indicates the site of a *Mutator* 6/7 transposon insertion into the *Rs1-1025* allele. Filled triangles indicate the approximate locations of *Mutator*-induced insertions/rearrangements associated with reversion of the *Rs1-O* phenotype. (B) *Bam*HI; (E) *Eco*RI; (H) *Hind*III; (S) *Sst*I; (X) *Xba*I.

and deletion of the *Rs1-O* locus, with *Rs1-O* phenotypic reversion, provide direct proof that the cloned homeo box gene is *rs1*.

Supporting evidence that the cloned homeo box sequence encodes the *rs1* gene comes from the isolation of the *Rs1-1025* allele. This allele arose independently in a stock containing *Mutator* transposons. The *Rs1-1025* mutant phenotype is very similar to *Rs1-O* and segregates with the *rs1* gene on chromosome 7S (Becraft and Freeling 1994). A λ genomic clone of the *Rs1-1025* allele was isolated and shown to contain a *Mu6/7* element (Chandler and Hardeman 1992) inserted into the third intron of the *rs1* gene, confirming that this sequence is responsible for the *Rough sheath 1* phenotype (Fig. 4).

The rs1 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 *rs1* sequence obtained from the 2-kb *Sst*I *rs1* genomic clone pB1. The longest clone contained ~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 λ genomic clone, λ8B73, isolated from a B73 inbred DNA library by hybridization with pB1 (Figs. 4 and 5). The genomic organization of the *rs1* gene is shown in Figure 4. Sequence data from λ8B73 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 5. The nucleotide sequence and predicted amino acid sequence of RS1. 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.

trons 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 *rs1* transcription unit and the deduced amino acid sequence of the RS1 protein. The longest open reading frame codes for an *M_r* 38,878 protein that contains a homeo domain. Translations in all three reading frames revealed no translation start signals in the genomic sequence 480-bp upstream of the first ATG in Figure 5. In addition, hybridization probes up to 3 kb upstream of the TATA box 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 *rs1* 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 *rs1* is almost identical to *kn1* and to *knt1* and 2 of *Arabidopsis* (see Fig. 6; Lincoln et al. 1994). The only difference between *rs1* and *kn1* splice site placement is intron III, which occurs 21 nucleotides 5' of the *kn1* site. *knt1* 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 site, 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

Figure 6. Comparison of the RS1 and KN1 amino acid sequences. Identities and conserved residues are indicated by bars and colons, respectively. The homeo domain is outlined, and the placement of the helix-turn-helix motif is shown above the 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 rs1 transcript is ectopically expressed in leaves of Rsl mutants

The wild-type pattern of *rs1* 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). Rehybridiza-

Figure 7. Gel blot of RNA from various immature organs of wild-type plants. The blot was hybridized with a near full-length *rs1* cDNA probe pVM4.1, and then reprobbed with a ubiquitin probe to control for loading. *rs1* transcript is abundant in roots, mesocotyl (the first internode and node of a seedling), ear and tassel, but not in leaf blades or sheaths. Poly(A)⁺ RNA (1.5 µg) was loaded per lane. The asterisk identifies a large, potentially unspliced precursor, transcript in the ear lane.

Figure 8. RNA gel blot analysis of wild-type and *Rs1-O* mutants. (A) RNA gel blots of poly(A)⁺ RNA extracted from whole seedling shoots of wild-type inbred B73, homozygous *Rs1-O*, heterozygous *Rs1-1025*, and homozygous *Rs1-Z4* plants. The blot was probed with the *rs1* cDNA EC1. The different alleles show no detectable transcript size difference. Per lane, 3.5 µg of RNA was loaded. (B) Gel blot of RNA from the ligular regions of early and late wild-type B73 leaves, and leaves displaying mutant phenotypes of *Rs1-O*, *Rs1-1025*, and *Rs1-Z4* heterozygotes. Early and late wild-type leaves were included because *Rs1-Z4* affects early leaves and *Rs1-O* and *Rs1-1025* affect late leaves. *rs1* transcript was detected in all three mutants but not in wild type, suggesting that the mutants ectopically express *rs1* transcript. The blot was reprobbed with actin, to confirm the presence of RNA in all lanes. Per lane, 1.5 µg of poly(A)⁺ RNA was loaded.

tion 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-

gions 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.]

involved in, or respond to, a pattern forming event that 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 *Rsl-O* shoot apices with normal siblings shows that *Rsl* 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 *Rsl-O* apex (Fig. 9H,J) shows more extensive and uniform *Rsl-O* expression than the wild-type. Interestingly, the midvein does not show ectopic expression and appears outlined by *Rsl* ectopic expression in neighboring cells (Fig. 9J). 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 *Rsl* interferes with normal ligule development at a stage that coincides with ligule initiation (Sylvester 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

Figure 10. RNA gel blot analysis of *Rsl-Or90* and *Rsl-Or122* revertant homozygotes. (A) RNA gel blot of 25 µg of total RNA isolated from ear primordia of *Rsl-O r90* and *Rsl-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.

leaves and is more uniform around the midvein region of the developing leaf (Figure 9J). In longitudinal section of mutants, the *Rsl* 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). *Rsl* 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 *Rsl* 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 *Rsl* may be involved in the specificity of the *Rsl* mutant phenotype for the ligular region of the leaf.

Phenotypic analysis of *Rsl-O* revertants

To understand the normal function of the *rs1* gene, self-fertilized progeny from *Mutator*-induced *Rsl-Or90*, *r122*,

Figure 9. In situ hybridizations of digoxigenin labeled *rs1* probe to sectioned normal and *Rsl-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 *Rsl* mutants. (H,I) Longitudinal sections through *Rsl-O/+* vegetative shoot meristem and immature blade regions of p4-7 leaves, respectively. The *rs1* transcript is ectopically expressed in young developing leaves and expression is increased in the shoot axis and meristem of *Rsl-O/+* plants compared to wild type. (J–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 *Rsl-O/+* shoot in J. (M) Meristem; (IM) inflorescence meristem. P1, 2, and 3 refer to Plastochron 1, 2, and 3 leaves. Bars, 100 µm.

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 reversions 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; Quaedyvlieg 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; Quaedyvlieg et al. 1995). The *glabra2* [*gl2*] gene represents a distinct class of homeo box genes that functions in trichome development. Recessive mutations of *gl2* re-

sult 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 Szymkowiak 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 subtend 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 *rs1* have a molecular role in the repeated ontogeny of plant organs.

Both *Rs1* and *Kn1* mutants impose sheath-like characteristics on blade regions of the leaf, yet unexpectedly, neither gene is normally expressed in the sheath. In *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 *Arabidopsis* and *Antirrhinum* act similarly (Bowman et al. 1989; Coen and Meyerowitz 1991; Drews et al. 1991; Mizukami and Ma 1992; Bradley et al. 1993). *rs1* and *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 *Drosophila* (Chan and Mann 1993).

There are several possible explanations for how ectopic expression of *Rs1* and *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 *rs1* and *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). *Rs1* and *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 *Rs1* and *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 *Kn1* affect primarily lateral veins of the blade, whereas *Rs1* alleles affect the whole ligular region and base of the blade (Freeling and Hake 1985; Freeling 1992; Hake 1992; Becraft and Freeling 1994). *Lg3* and *Lg4* mutants have other distinct specificities (Fowler 1994). Ectopic expression of *kn1* in the veins predicts the placement of mutant cells in *Knotted* leaves (Smith et al. 1992), however, expression patterns do not fully explain the tissue-specific mutant phenotype of *Rs1-O*. The region of ectopic expression of *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 *Mutator* transposon-induced revertants showed recurrent *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 *Mutator* transposon insertions in different regions of the gene (Fig. 4). Segregation analysis of the *Rs1-Or90*, *r122*, and *r312* alleles showed that only *Rs1-Or312* was closely linked to a recessive embryo lethal phenotype. The *Rs1-Or312* allele may be *Mutator* suppressible and require *Mutator* activity to effect the mutant phenotype (Barkan and Martienssen 1991). The *Rs1-Or90* and *r122* alleles still express *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 non-coding 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 *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 *Drosophila* where the *Ultrabithorax* and *abdominalA* genes nonautonomously induce the expression of *labial* in cells adjacent to their expression domains (Immergluck et al. 1990; Reuter et al. 1990). This nonautonomous regulation requires the action of *decapentaplegic*, which encodes a TGF- β -like protein (Padgett et al. 1987) that is the apparent inducing signal. *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

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 μ l of TE, and 15 μ l was used per restriction enzyme digest. The DNA was digested with *Bam*HI, *Eco*RI, *Hind*III (New England Biolabs), or *Sst*I (GIBCO BRL) using the manufacturer's buffers. The restricted DNA was electrophoresed through 0.8% agarose gels and transferred to

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 (NEN) with a Prime It random primer kit (Stratagene). The 247-bp *kn1* 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

*Sst*I-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 *Sst*I-digested λZap (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 *kn1* 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 λZap in vivo excision method (Stratagene).

The 2-kb *Rs1-O* *Sst*I fragment was used as a probe to isolate an ~18-kb *Eco*RI fragment from the commercial inbred line B73 (λ8B73 in Fig. 4) and a 9-kb *Eco*RI fragment from the *Rs1-1025* mutant. DNA from each line was digested with *Eco*RI and enriched for the respective fragment sizes by sucrose gradient fractionation. Libraries were constructed by ligation of the genomic fragments into *Eco*RI predigested λDASH (Stratagene). The library was packaged with Gigapack packaging extracts (Stratagene) and plated onto SRBP2 cells. Plaque lifts were screened at high stringency with the *rs1* 2-kb *Sst*I fragment.

cDNA isolation

Four cDNA clones, EC1, EC6, EC20, and EC21 were kindly provided by Bruce Veit as *kn1* 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'-accattcgggtccatgtcatc-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 λZAPII 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 (NEN), 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 an ear *rs1* cDNA from which the sequence 3' from the *Sph*I 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° 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 Merckoglass (EM Science). Competition experiments with an unlabeled *kn1* probe showed no change in signal localization or intensity, indicating that the probe is specific for *rs1* transcripts.

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