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Sequence Analysis and Expression Patterns Divide the Maize *knotted1*-like Homeobox Genes into Two Classes

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The homeobox of the *knotted1* (*kn1*) gene was used to isolate 12 related sequences in maize. The homeodomains encoded by the *kn1*-like genes are very similar, ranging from 55 to 89% amino acid identity. Differences outside the precisely conserved third helix allowed us to group the genes into two classes. The homeodomains of the seven class 1 genes share 73 to 89% identical residues with *kn1*. The four class 2 genes share 55 to 58% identical residues with *kn1*, although the conservation within the class is greater than 87%. Expression patterns were analyzed by RNA gel blot analysis. Class 1 genes were highly expressed in meristem-enriched tissues, such as the vegetative meristem and ear primordia. Expression was not detectable in leaves. The class 2 genes were expressed in all tissues, although one was abundantly expressed in roots. The genes were mapped using recombinant inbred populations. We determined that clusters of two to three linked genes are present on chromosomes 1 and 8; otherwise, the genes are distributed throughout the genome. Four pairs of genes, similar in both sequence and expression patterns, mapped within duplicated regions of the genome.

INTRODUCTION

The homeobox was first identified as a cross-hybridizing DNA sequence shared among several genes that control morphogenesis in Drosophila (McGinnis et al., 1984; Scott and Weiner, 1984). Comparison of the deduced amino acid sequences from these genes revealed a region with similarity to known regulatory proteins encoded by the yeast mating type loci *MATα2* and *MATa1* (Laughon and Scott, 1984; Shepherd et al., 1984). This conserved protein motif became known as the homeodomain. Subsequently, the homeobox has been found in a number of genes that control cell fate or convey positional information during the development of diverse groups of animals, fungi, and plants (for review, see Gehring, 1987; Scott et al., 1989; Kessel and Gruss, 1990).

Homeodomain proteins are thought to act as sequence-specific DNA binding proteins that can directly regulate the expression of specific groups of target genes (Affolter et al., 1990a; Hayashi and Scott, 1990). Although the primary amino acid sequences show relatively low identity, 27%, high-resolution structural studies of the homeodomains of *Engrailed* (*En*) and *Antennapedia* (*Antp*) and of α2 complexed with DNA have revealed nearly identical structures (Kissinger et al., 1990; Otting et al., 1990; Wolberger et al., 1991). The homeodomain of each protein contains three α-helices, although the third α-helix of ANTP is kinked and is therefore referred to as helices 3 and 4 (Qian et al., 1989). Residues in helix 3, dubbed the "recognition" helix, make sequence-specific contact in the major groove of the DNA binding site, and basic residues of the N-terminal portion of the homeodomain make contact in the minor groove forming a pincer-like grip. The remarkable conformational identity between distantly related homeodomains suggests that important structural and functional properties are shared among all members of this diverse class of genes (Kornberg, 1993).

Although structurally similar, different homeodomains are able to recognize diverse DNA binding sites. This has been clearly illustrated by experiments that show that changes of as few as one residue in the third helix can dramatically alter the specificity of DNA binding (Hanes and Brent, 1989; Treisman et al., 1989; Affolter et al., 1990b; Percival-Smith et al., 1990). Different homeodomain proteins have been grouped into separate families or classes based on either sequence identity within the homeodomain or conserved protein motifs outside of the homeodomain (Scott et al., 1989; Treisman et al., 1991). Examples of such families of homeodomain proteins in animal systems include the Antennapedia, Engrailed, Paired, POU, and LIM classes. The Antennapedia class is a large family; its members comprise the homeotic complexes (HOM-C in Drosophila and Hox in vertebrates; for review, see Krumlauf, 1992). The distinguishing feature of this class is an identical core sequence of 12 amino acids in the recognition helix; this

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sequence is shared by all members. The POU class of homeodomain proteins, in contrast, is distinguished by the presence of a 75–amino acid POU domain separated from the homeodomain by 20 to 30 amino acids (Herr et al., 1988). In plants, four different types of homeodomain proteins have been described thus far: homeodomain zipper proteins (HD-Zip), which are distinguished by the presence of a leucine zipper adjacent to the homeodomain (Ruberti et al., 1991; Mattsson et al., 1992; Schena and Davis, 1992, 1994); plant homeodomain finger proteins (PHD-finger) represented by ZMHOX1A in maize (Bellmann and Werr, 1992), HAT3.1 in Arabidopsis (Schindler et al., 1993), and the pathogenesis-related homeodomain proteins in parsley and Arabidopsis (PRHP and PRHA, respectively; Korfhage et al., 1994), which share a conserved cysteine-rich motif; the Arabidopsis GLABRA2 homeodomain protein (Rerie et al., 1994); and KNOTTED1-like proteins (Vollbrecht et al., 1991; Matsuoka et al., 1993; Ma et al., 1994), which are the subject of this report.

The first family of homeobox genes reported in a plant species was isolated by transposon tagging the morphological mutation *Knotted1* (*Kn1*) in maize (Hake et al., 1989; Vollbrecht et al., 1991). *Kn1* is one of a number of dominant mutations that alter the developmental fate of cells in the leaf blade toward sheath-like identities (for reviews, see Freeling, 1992; Hake, 1992). Wild-type expression of *kn1* is localized to the meristem and ground tissue of the unexpanded stem and is absent from leaves and leaf primordia. In addition, *kn1* is expressed throughout the corpus of the inflorescence meristem and in spikelet and floral meristems but is not expressed in lateral organs such as glume primordia (Smith et al., 1992; Jackson et al., 1994). In the dominant *Kn1* mutants, however, ectopic expression of *kn1* in the veins of the developing leaf blade has been correlated with the mutant phenotype (Smith et al., 1992). These gain-of-function mutations typically affect the lateral veins, causing the adjacent tissues to differentiate incompletely and continue dividing, with the result that sporadic outgrowths, or knots, are formed on the leaf blade (Gelinas et al., 1969; Freeling and Hake, 1985). Transgenic tobacco plants expressing the *kn1* cDNA driven by the cauliflower mosaic virus 35S promoter have altered leaf size and shape, and in severe cases, ectopic shoots arise from the leaf surface (Sinha et al., 1993). The results suggest that ectopic expression of *kn1* causes leaf cells to adopt different fates that range from alternative types of leaf cells to meristem cells (Smith et al., 1992; Sinha et al., 1993).

In animals, families of related homeobox genes often specify related developmental processes (Gehring, 1987; Krumlauf, 1992). By taking advantage of the conserved homeobox, we isolated a number of *kn1*-like genes from maize (Vollbrecht et al., 1991, 1993). If members of this class of genes specify related developmental processes, they may, like *kn1*, play significant roles in the development of higher plants. Here, we compare the deduced amino acid sequences of the *kn1*-like genes in the homeodomain, characterize their patterns of steady state mRNA expression, and report their chromosomal locations on the maize genetic map.

RESULTS

Isolation and Sequencing of *knox* Genes

To assess the approximate number of *kn1*-like homeobox genes present in the maize genome, a maize genomic DNA gel blot was prepared and hybridized at low stringency to a probe consisting of only the homeobox (see Methods). The blot was stripped and reprobed at high stringency with a gene-specific probe from the third intron of *kn1*. Figure 1 shows that while the gene-specific probe hybridizes to only one band (Figure 1B), the homeobox probe hybridizes to 10 to 15 sequences in the maize genome at low stringency (Figure 1A). The large number of bands suggests that the homeobox in *kn1* represents one member of a fairly large gene family. We propose the name *knox* (for *knotted*-like homeobox) to distinguish this gene family from other types of homeobox genes.

Genomic and cDNA libraries were screened with the *kn1* homeobox–specific probe at low stringency. After several new clones were isolated and characterized, the libraries were

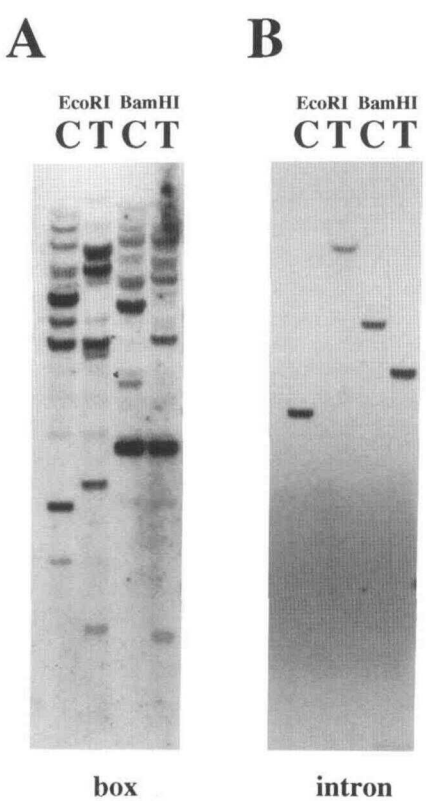
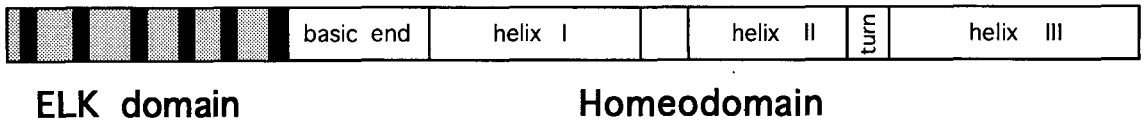


Figure 1. Maize Genomic DNA Gel Blot Analysis.

(A) Genomic DNA from the inbred maize lines CM37 (C) and T232 (T), digested with either EcoRI or BamHI, was run on an agarose gel, blotted, and probed with the *kn1* homeobox (box) at low stringency. **(B)** The blot in **(A)** was stripped and reprobed with a gene-specific probe from the third intron of *kn1* at high stringency.

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KN1     VDQELKHHLLKKYSGYLSSLKQEL SKKKKKGKLPKEARQQLLSWWDQHYKWPYPSETQKVALAESTGLDLKQINNWFINQRKRHWKPS
RS1     E-K---YQ------------R--F ----------------K--H--EL----------E-I---------Q-----------------
KNOX4   ...---YQ------------R--F ----------------K--H--EL----------E-I----A----Q-----------------
KNOX8   ...----Q--R--G---GG-R--F --R------------K--H--EL----------E-M----T----P----------------A
KNOX3   D-K---KQ--R----C-GN-RK-- C--R--D---------K-----EL--R------ME-I---------EQ-----------------
KNOX10  E-ND--NR--N---------WR-- -R---------RD---K--H--QL--R------LE-A---------EA----------------QA
LG3     ...---EM-------C--R-RS-F L--R-------D--TV--E--NT--R----T-ED--R--AM----P-----------------
KNOX5   ...---EM-------C--R-RS-F L--R-------D--SA-MD--NT--R----T-ED--R--AM----P-----------------
KNOX11  A-R---EM-------C--R-RS-F L--R-------D--SA-MD--NT--R----T-ED--R--AA----P-----------------

KNOX1   -R----LE-KQGFKSRIEDVRE-I LR-RRA----GDTTSI-KQ--QE-S-----T-DD-AK-V-E---Q-------------N-HNN
KNOX2   -R----HE-KQG-RDK-VDIRE-I LR-RRA----GDTAST-KA--QA-S-----T-ED-AR-VQE---Q-------------N-HNN
KNOX6   -RK---NE-KQG-KEK-VDIRE-I MR-RRA----GDTASV-KA--QA-S-----T-DD-AR-VQE---Q-------------N-HSN
KNOX7   ...---NE-KQG-KEK-VDIRE-I MR-RRA----GDTASV-KA--QA-S-----T-DD-AR-VQE---Q-------------N-HSN
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Figure 2. Alignment of KNOX Homeodomain Sequences.

Deduced amino acid sequences were aligned with the homeodomain of KN1 (identical residues are indicated with dashes). Class 1 sequences are separated from class 2 sequences by a space. Features of the homeodomain and flanking ELK domain are shown in the diagram at the top. Irregularly repeating hydrophobic residues conserved in the ELK domain are represented by thick black stripes on a stippled bar. Intron positions are indicated by arrows; the solid arrow indicates the position of an intron conserved in all members of the family, and an open arrow indicates the position of an intron in class 2 genes. *knox1* and *knox2* have been previously reported as *zmh1* and *zmh2* (Vollbrecht et al., 1991).

screened again at low stringency with a mixture of different homeobox probes to isolate as many cross-hybridizing clones as possible. The cDNA libraries were prepared from poly(A)$^+$-enriched RNA isolated from seedlings and from ear primordia (Schmidt et al., 1993). Clones that also hybridized to the *kn1* cDNA at high stringency were discarded, and the rest were sorted by polymerase chain reaction and DNA sequencing. The clones were initially sequenced using degenerate oligonucleotides corresponding to the third helix of the homeobox. Sequence data from degenerate oligonucleotides permitted the design of specific oligonucleotides for further sequencing. Clones corresponding to 12 different loci were isolated, and for six, both genomic and cDNA clones were found.

Each of the clones was sequenced in the homeobox region. All the deduced homeodomains depicted in Figure 2 show considerable similarity to that of KN1. The amino acid identity to KN1 ranges from 89 (ROUGH SHEATH1 [RS1]) to 55% (KNOX6 and KNOX7). The nucleotide identity to the *kn1* homeobox ranges from 84 (*knox4*) to 56% (*knox7*). A stretch of 13 amino acids in the third helix, the putative recognition helix, is precisely conserved in all the deduced proteins. A cluster of basic residues is found at the N-terminal end of each homeodomain. This region, by analogy with the corresponding region of the Engrailed protein, may make contact with DNA in the minor groove (Kissinger et al., 1990). All the homeodomains carry a three–amino acid insertion between the first and second helix, similar to an insertion found in the α2 homeodomain. Amino acid differences in other parts of the homeodomain were used to group the genes into two broad classes. The eight genes encoding homeodomains most similar to that of KN1, including *rs1*, *knox3*, *knox4*, *knox8*, *knox10*, *liguleless3* (*lg3*), *knox5*, and *knox11*, will be referred to as class 1 *knox* genes; their amino acid identity with KN1 in the homeodomain ranges from 73 to 89%. The others, *knox1*, *knox2*, *knox6*, and *knox7*, encode homeodomains that share only 55 to 58% identity with KN1 but greater than 87% identity with each other; they will be referred to as class 2 *knox* genes.

All 12 of the *knox* genes showed an additional region of sequence similarity immediately adjacent to and upstream of the homeodomain (Figure 2). Dubbed the ELK domain (Vollbrecht et al., 1991) for a highly conserved series of Glu, Leu, and Lys amino acids, the motif spans ~24 amino acids and extends the conserved block (ELK plus homeodomain) to ~88 amino acids. Distinguished by highly conserved leucine or other hydrophobic residues interspersed in a region of predominantly charged or polar residues, the repeating leucine motif is reminiscent of a leucine zipper (Landschulz et al., 1988), but the periodicity of hydrophobic residues is distinct from other amphipathic helices (Murre et al., 1989; Alberti et al., 1993). The hydrophobic residues are repeated at intervals of four and five amino acids; this may result in a novel form of amphipathic helix with an offset hydrophobic face. The ELK motif is found in the protein encoded by the *kn1* homolog from rice, *OSH1* (Matsuoka et al., 1993), and by *kn1*-like genes of Arabidopsis (Lincoln et al., 1994), soybean (Ma et al., 1994), and tomato (N. Sinha and S. Hake, unpublished data).

Each of the *knox* genes has an intron in an absolutely conserved position within the homeobox near the N-terminal end of the second helix (solid arrow, Figure 2). In addition, the *knox* genes in the class 2 subgroup share another small intron in the adjacent ELK region (open arrow, Figure 2). The positions of the introns interrupting the plant homeoboxes are distinct from the positions of introns reported in animal or fungal homeoboxes (for a catalog of homeobox intron positions, see Allen et al., 1991).

Analysis of *knox* Gene Expression

kn1 is highly expressed in the vegetative meristem and inflorescence primordia of maize and undetectable in more determinate organs, such as leaves, roots, and floral organs (Smith et al., 1992). To investigate *knox* gene expression in plant tissues, both total RNA and poly(A)$^+$-enriched RNA were isolated from various maize tissues. RNA blots were prepared using 1 μg of poly(A)$^+$-enriched RNA per lane and were hybridized with DNA probes from each of the *knox* genes for which cDNA clones were isolated. All the *knox* genes examined by hybridization to RNA blots detected a single band between 1.5 and 1.7 kb in length. The class 1 genes *kn1*, *rs1*, *knox3*, *knox4*, and *knox8* were all found to be strongly expressed in ear inflorescence primordia and shoot meristem–enriched tissue, moderately to weakly expressed in embryos, and not detected in leaves, as shown in Figure 3. A subset, *rs1* and *knox4*, were weakly expressed in roots. The expression of *knox5* was similar to *rs1* and *knox4* on total RNA blots (data not shown), and *knox10*, *knox11*, and *lg3* were not extensively tested because of the inadequacy of the probes available for use in RNA gel blot analysis.

By contrast, the class 2 *knox* genes showed greater diversity in their patterns of expression. The *knox1* gene was weakly expressed in all tissues (data not shown) and strongly expressed in roots. *knox2* appeared to be expressed at a significant level in all tissues examined, although its expression level was strongest in leaves. *knox6*, like *knox2*, was strongly expressed in all the tissues examined but showed somewhat stronger expression in meristematic tissues. The pattern of *knox7* expression was indistinguishable from that of *knox6* (data not shown). Accurate quantitative comparisons between signals on poly(A)$^+$-enriched RNA from different tissues were difficult because of potential sample-to-sample variation in the efficiency of poly(A)$^+$ enrichment. In practice, the patterns of expression seen with 1 μg of poly(A)$^+$-enriched RNA shown in Figure 3 closely mirrored the patterns seen with 10 μg of total RNA (data not shown).

RNA gel blot hybridizations were carried out at high stringency to prevent cross-hybridization of related mRNAs. Each probe was shown to be specific by preparing slot blots containing RNA synthesized with T3 polymerase from each of the other KNOX clones. Duplicate slot blots were then hybridized with probes derived from each KNOX clone. With 0.01 ng of RNA loaded per slot, self-hybridization was at least 100-fold greater than hybridization to any other clone (data not shown). The KNOX4 probe is an exception. It was generated from a short genomic clone and showed only ~20-fold greater hybridization to itself than to the RS1 cDNA clone; although in the reciprocal hybridization, the RS1 cDNA probe did not hybridize significantly to the KNOX4 clone (data not shown). Differences in the patterns of expression and minor differences in the sizes of the bands revealed with different *knox* gene probes also indicate that cross-hybridization was not significant.

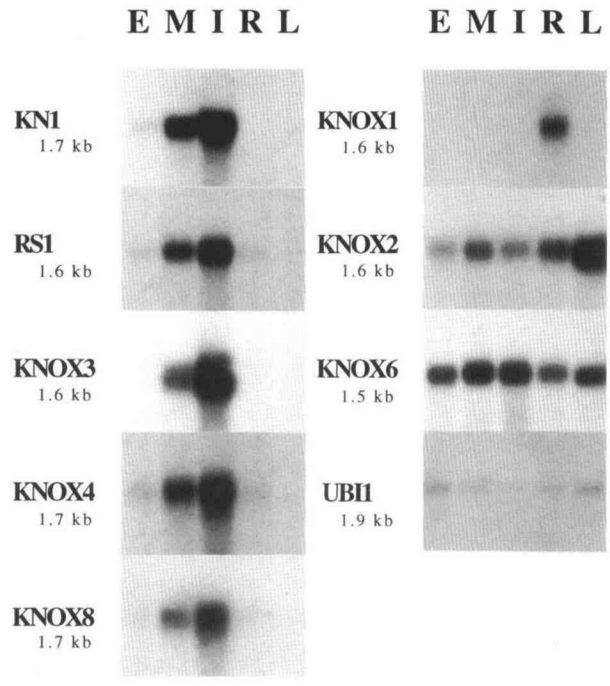

Figure 3. RNA Gel Blot Analysis of *knox* Genes.

RNA gel blots were prepared from 1 μg each of poly(A)$^+$-enriched RNA collected from embryos 17 days postpollination (lanes E), shoot meristems including unexpanded stem and the youngest leaf primordia (less than 0.5 cm) (lanes M), ear inflorescence primordia up to 2 cm long (lanes I), primary roots from seeds germinated in air (lanes R), and the blade portion of fully expanded juvenile leaves (lanes L). Blots were probed with gene-specific probes for a subset of class 1 *knox* genes (left) and class 2 *knox* genes (right). The approximate length of each transcript is indicated below the gene designation. Reprobing with the maize ubiquitin cDNA (UBI1) allowed the quantity of RNA in each lane to be compared.

Chromosomal Map Locations of *knox* Sequences

The *knox* genes were mapped on recombinant inbred lines according to the method of Burr and coworkers (Burr et al., 1988). Approximate map positions are shown in Figure 4. Chromosomes 2, 3, 4, 7, and 9 have one *knox* gene, chromosome 5 has two *knox* genes, and chromosomes 1 and 8 contain small clusters of three and two linked *knox* genes, respectively. Only

chromosomes 6 and 10 remain without any mapped *knox* gene. Four pairs of closely related but unlinked *knox* genes (*knox1* and *knox2*, *knox6* and *knox7*, *knox4* and *rs1*, and *lg3* and *knox5* or *knox11*) map in regions of the genome that share a number of duplicate genes (Helentjaris et al., 1988).

At least three *knox* genes map to the long arm of chromosome 1. Within the resolution of the CO159 × Tx303 recombinant inbred mapping population, *knox3* maps to the same location as *kn1*, and *knox8* resides ~14 centimorgans distal to *kn1* and *knox3*. To examine further the linkage of *kn1* and *knox3*, we took advantage of a deletion that completely removes the *kn1* locus but not the closely linked markers *alcohol dehydrogenase1* and *lemon-white*. The deletion, *def(kn1)-O*, is not transmitted through the male gametophyte (Hake et al., 1989; Veit et al., 1990) and results in a defective embryo when uncovered by a much larger deficiency (L. Smith and S. Hake, unpublished data). In Figure 5, a gene-specific probe for *knox3* detects a restriction fragment length polymorphism (RFLP) between the progenitor of the deletion (*Kn1-O*) and a wild-type line (+). We crossed the heterozygote carrying *Kn1-O* on one chromosome and

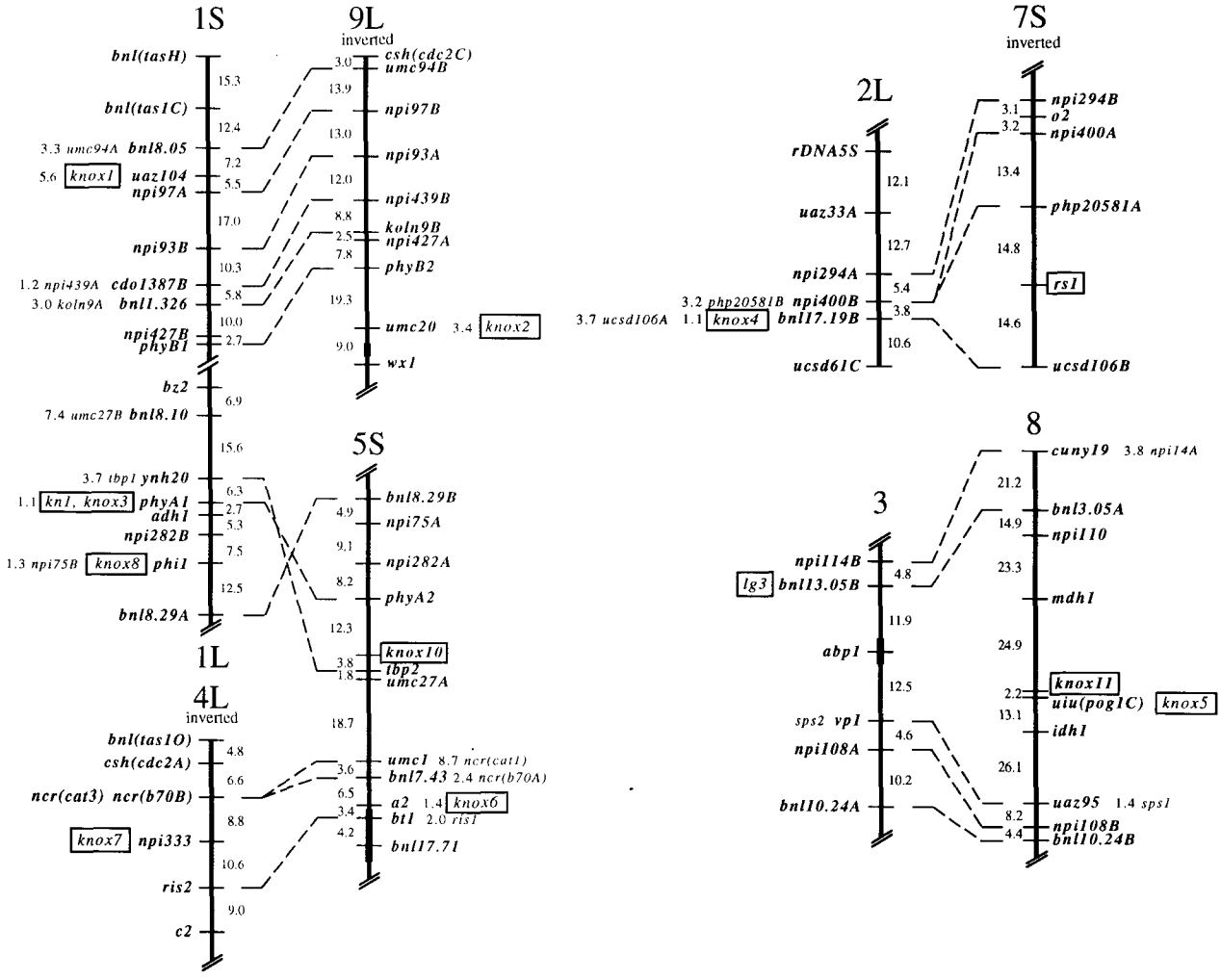

Figure 4. Genome Map Positions of *knox* Genes.

knox gene sequences were mapped on recombinant inbred lines according to the method of Burr et al. (1988). Segments of chromosomes or chromosome arms (S, short arm; L, long arm) are represented by solid lines, with centromeric regions indicated by a thickened line. Map distances are in centimorgans. Framework loci are shown in bold; loci in normal type on the same line are linked to the nearest framework locus by the distance indicated. RFLP markers and distances, taken from the current genome map maintained by E. Matz, F. Burr, and B. Burr (personal communication), convey the linkage relationships of the *knox* genes. The dashed lines connect two RFLP loci that hybridize to a single probe and may reflect ancient duplications of regions of the genome (Helentjaris et al., 1988). The pairs of genes *knox6* and *knox7*, *lg3* and *knox11* (or *knox5*), and *knox4* and *rs1* are similar in sequence and expression and fall within these duplicated regions. Some map segments were inverted from the conventional orientation to simplify comparisons between putative duplicated regions.

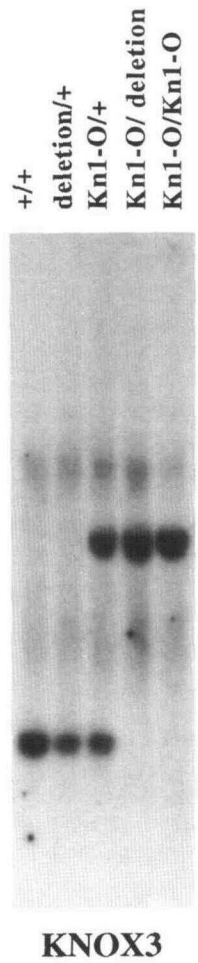

Figure 5. A Deletion of *kn1* Also Removes *knox3*.

The *knox3* probe detected a polymorphism between *Kn1-O*, the progenitor of a deletion that completely removes the *kn1* locus, and a wild-type allele (+) when DNA was digested with BamHI. The probe detected only the (+) polymorphic band in DNA of plants carrying both the deletion and the wild-type (+) allele (lane marked deletion/+). The absence of a *knox3* band associated with the deletion chromosome suggests that this locus is also missing in the deletion. Genotypes at the *kn1* locus are indicated at the top of each lane: + indicates a wild-type allele, deletion is *def(kn1)-O*, and *Kn1-O* is a dominant mutant allele and progenitor of *def(kn1)-O*.

def(kn1)-O (deletion) on the homologous chromosome by the wild-type line and analyzed the DNA of the knotted and normal progeny. Both RFLPs were clearly observed in the knotted (*Kn1-O*/+) progeny; however, the normal progeny (*def(kn1)-O*/+) showed only the wild-type RFLP. The absence of a *knox3* band associated with the deletion chromosome suggests that the locus is also missing in the deletion, although the precise extent of the deletion remains uncertain.

DISCUSSION

The *kn1* class of homeobox genes in maize (*knox* genes) comprise a family of ~13 genes that map to eight of the 10 chromosomes. The genes all contain a conserved intron position and a three–amino acid insertion within the homeodomain. The third helix of the homeodomain is invariant. The similarity extends outside the homeodomain to include an adjacent region, the ELK domain, that contains repeating hydrophobic residues. The family can be divided into two subclasses based on sequence similarity within the homeodomain and patterns of expression at the level of RNA gel blots. The first class is more like KN1, sharing 73 to 89% amino acid identity in the homeodomain and being expressed primarily in shoot and inflorescence meristems but not in leaves. The second class differs from KN1 in the extent of sequence similarity and pattern of expression. These proteins share only 55 to 58% identity with KN1 in the homeodomain, and they appear to be expressed at varying levels in all tissues, including mature leaves.

kn1-like homeobox genes have recently been reported in rice (Matsuoka et al., 1993) and identified in several dicot species, including Arabidopsis (Lincoln et al., 1994), tomato (N. Sinha and S. Hake, unpublished data), and soybean (Ma et al., 1994). The sequence similarity within the homeodomain, regardless of species, is high. Sequence conservation among *kn1*-like genes across monocot and dicot plant species suggests that there is functional significance that has been conserved over evolutionary time. As shown in Figure 6, other classes of plant homeodomains are more divergent in sequence. Most of these other proteins are also distinguished by conserved features outside of the homeodomain, such as leucine zippers in the HD-Zip proteins (Ruberti et al., 1991; Mattsson et al., 1992; Schena and Davis, 1992, 1994) and putative metal binding domains in the PHD-finger proteins (Bellmann and Werr, 1992; Schindler et al., 1993; Korfhage et al., 1994). The recognition helix of the GLABRA2 protein in Arabidopsis is somewhat similar to the HD-Zip class of homeodomains, but the protein lacks a leucine zipper, so it represents a distinct class of plant homeodomain protein.

Mapping of the *knox* genes on the maize genome reveals several interesting features. Whereas two or three genetically linked homeobox genes are found on chromosomes 1 and 8, there is no indication of extensive clusters of homeobox genes as has been observed with the HOM-C or Hox clusters of homeobox genes in animal genomes (Krumlauf, 1992). The closely linked maize genes encode similar homeodomains and may be the result of small duplications that have subsequently diverged.

A number of researchers have shown that the maize genome contains large numbers of duplicated genes that are distributed in a nonrandom manner such that certain pairs of chromosomes share many duplicated genes (Wendel et al., 1986; Helentjaris et al., 1988). Helentjaris and coworkers reported

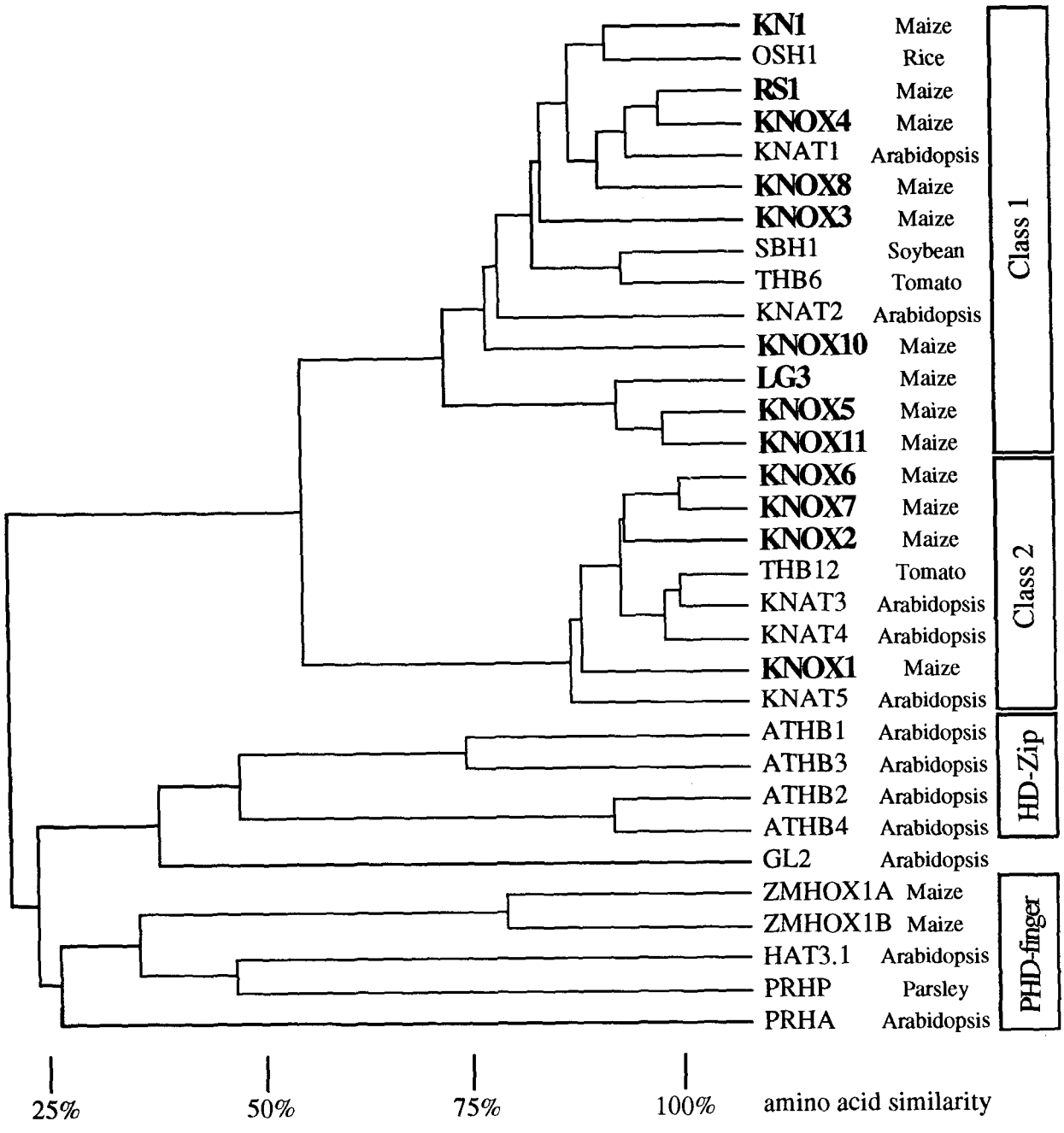

Figure 6. Analysis of Plant Homeodomain Sequence Similarity.

The dendrogram reflects the degree of similarity between deduced amino acid sequences from various plant homeodomains using the unweighted pair group maximum averages method (Sneath and Sokal, 1973). Percent similarity between homeodomain sequences is indicated by the scale at the bottom. The maize *kn1*-like homeodomains are in bold type. Homeobox genes similar to *kn1* from other species include *OSH1* from rice (Matsuoka et al., 1993), *sbh1* from soybean (Ma et al., 1994), the *KNAT* genes in Arabidopsis (Lincoln et al., 1994), and *thb6* and *thb12* from tomato (N. Sinha, J. Mathern, and S. Hake, unpublished data). The two classes of KNOX homeodomains are easily distinguished and appear to have diverged prior to the separation of monocots and dicots. The other types of homeobox genes reported in plants fall into completely different classes: the HD-Zip class; *Athb-1*, *Athb-2* (Ruberti et al., 1991; these homeodomains have the same sequence as *HAT5* and *HAT4*, respectively; Schena and Davis, 1992), *Athb-3* (Mattsson et al., 1992), and *Athb-4* (Carabelli et al., 1993); the *GLABRA2* (*GL2*) class (Rerie et al., 1994); the PHD-finger class; *ZMHOX1A*, *ZMHOX1B* (Bellmann and Werr, 1992), *HAT3.1* (Schindler et al., 1993), *PRHP*, and *PRHA* (Korfhage et al., 1994).

that ~29% of the 200 single-copy RFLP probes that they used detected an unlinked, duplicate sequence. Because related duplications are found in other grass species, such as sorghum (Berhan et al., 1993), the modern maize genome may be the result of an ancient duplication or allopolyploidy that occurred early in the evolution of the grass supertribe Andropogonodae. Certain pairs of *knox* genes, such as *knox4* and *rs1*, show significantly more similarity to each other than to the rest of the gene family and map within these duplicate regions (Figures 4 and 6). These results suggest that these *knox* gene pairs may have once been duplicate genes.

All of the KN1-like homeodomains examined in plants share an identical series of 13 amino acids in the third helix. This portion of the homeodomain is thought to be largely responsible for much of the sequence-specific contact with DNA (for review, see Kornberg, 1993). Identical recognition sequences may indicate that different proteins recognize the same set of downstream targets or that additional specificity is provided by differences in other parts of the homeodomain, by other domains of the proteins, or by interactions with other proteins that help to specify DNA recognition. Much attention has been given to the problem of specificity in target DNA recognition (for reviews, see Affolter et al., 1990a; Hayashi and Scott, 1990). It appears that amino acid sequences within the homeodomain, apart from the recognition helix, can determine target specificity or influence the ability of a particular protein to form a specific protein–DNA complex. In Drosophila for example, the homeotic genes *Antp* and *Sex combs reduced* (*Scr*) encode homeodomains that differ at only four residues in the N-terminal part of the homeodomain, yet they condition different phenotypes when expressed ectopically under the heat shock promoter (Zeng et al., 1993). Domain-swapping experiments have revealed that the N-terminal region of the homeodomain is sufficient to distinguish the functional specificity of the SCR protein from that of ANTP in vivo, although a few features are influenced by sequences outside of the homeodomain (Zeng et al., 1993). The mammalian octamer motif binding factors, Oct-1 and Oct-2, share little similarity apart from the POU and homeodomains, yet swapping experiments have demonstrated that the ability of Oct-1 to form a DNA binding complex with the herpes simplex virus *trans*-inducing factor, VP16, depends on only a few amino acid differences in helix 2 of the homeodomain (Stern et al., 1989). Clearly, the recognition helix may not be the only determinant of downstream target specificity. When combined with sequence differences that lie outside of the homeodomain that may mediate differential interactions with the transcriptional machinery or with specific *trans*-acting factors, there exists ample opportunity for proteins with quite similar homeodomains to regulate different sets of target genes specifically.

Expression of the five class 1 *knox* genes examined on RNA gel blots is high in meristem-enriched tissues and inflorescence primordia but not detectable in leaves. Because the meristem-enriched region includes the youngest leaf primordia, RNA gel blot analysis does not address when expression disappears during leaf development. In situ hybridization analysis has shown that expression in the meristem of *rs1*, *knox3*, and *knox8*, like *kn1*, is exclusive of leaf primordia (Jackson et al., 1994) even at the very earliest stages of leaf initiation. The staining patterns indicate overlapping but distinct domains of expression within the shoot apical meristem. *kn1* and *knox8* are expressed throughout the central region of the shoot apical meristem in a similar pattern, although the staining of *kn1* is stronger than that of *knox8*. By contrast, *rs1* and *knox3* expression is limited to a subset of meristem cells (Jackson et al., 1994). All four genes are also expressed in the unexpanded stem, and the patterns of expression are quite similar in the inflorescence primordium.

The *kn1* locus was originally defined by dominant mutations that affect leaf development (Bryan and Sass, 1941; Gelinas et al., 1969; Freeling and Hake, 1985). The mutations, which are either transposon insertions or rearrangements of 5′ sequences (Hake, 1992), result in ectopic expression of *kn1* in lateral veins of developing leaves (Smith et al., 1992). Localized proliferations of cells around lateral veins result in outgrowths or knots on the leaf blade. There is also an overall reduction in height of the plant and a broadening of the leaf. Cells along the lateral veins in the blade adopt fates of cells normally found in more basal regions of the leaf (Sinha and Hake, 1994). Transgenic tobacco plants expressing the maize *kn1* cDNA under the cauliflower mosaic virus 35S promoter are also affected in plant height and leaf shape in a dosage-dependent manner (Sinha et al., 1993). In the most severely affected plants, leaf cells adopt meristem fates and form ectopic shoots.

Two of the class 1 *knox* genes have been shown to correspond to existing morphological mutations that affect the maize plant in a manner similar to *kn1*. The dominant *Rs1* mutation (P. Becraft, R. Schneeberger, S. Hake, and M. Freeling, unpublished data) and the dominant *Lg3* mutation (J. Fowler and M. Freeling, unpublished data) correspond to homeobox genes that are closely related to *kn1* (Figures 2 and 6). Several other *knox* sequences show tight genetic linkage to known mutations, including *knox11* and *knox5* to *Liguleless4* (J. Fowler and M. Freeling, unpublished data) and *knox4* to the morphological mutation *Gnarley1* (T. Foster and S. Hake, unpublished data). All of these dominant mutations alter cell fates in the maize leaf in a manner analogous to *Kn1* but appear to affect different tissues specifically. For example, whereas *Kn1* primarily affects the lateral veins, *Rs1* affects the ligule/auricle region (Becraft and Freeling, 1994) and *Lg3* affects primarily the midrib region (for review of these mutations, see Freeling, 1992). Because the *Kn1* mutations result from ectopic expression in leaves where the gene is not normally expressed, it is likely that the dominant mutations of the other *kn1*-like genes also result from ectopic expression.

Although these class 1 genes are similar in sequence, expression patterns, and dominant phenotypes and in some cases map within regions that may have arisen as part of an ancient duplication, they are not necessarily redundant. Despite many examples of duplicate homeobox genes, gene redundancy may not be common. For example, in mice there are four copies of the *Hox* gene cluster located on four separate chromosomes (Hunt and Krumlauf, 1992). Targeted gene

disruptions of the paralogous genes from two different clusters, *Hoxd-3* and *Hoxa-3*, produce different phenotypes despite the fact that the genes have identical expression patterns (Chisaka and Capecchi, 1991; Condie and Capecchi, 1993). Recent data from our laboratory suggest that *kn1* is not redundant, as we have found a recessive inflorescence phenotype (R. Kerstetter, L. Smith, and S. Hake, unpublished data). Similar experiments are underway for related *kn1*-like genes (M. Freeling, personal communication). Exactly what role each *knox* gene plays in development and how their activities are coordinated await analysis of such recessive alleles. Examination of the patterns of expression and mutant phenotypes of the *kn1*-like homeobox genes in dicot species should also serve to elucidate further the role of these genes in plant development.

METHODS

DNA Isolation and Blot Hybridization

Maize genomic DNA was isolated as described by Chen and Dellaporta (1994). DNA (7 ug per lane) was digested with the appropriate endonucleases, separated on 0.8% agarose gels, and transferred to Magna nylon transfer membranes (Micron Separations Inc., Westboro, MA). Low-stringency hybridization was performed in 9 × SSC (1 × SSC is 0.15 M NaCl, 15 mM sodium citrate), 2 mM EDTA, 10 mM Tris, pH 7.5, 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), 50 μg/mL sheared salmon sperm DNA, 20 mM sodium phosphate, pH 7, 2% SDS at 60°C, and filters were washed in 2 × SSC, 0.2% SDS twice for 1 hr at the same temperature. Normal high-stringency genomic DNA gel blot hybridizations were performed in a hybridization oven (Robbin's Scientific, Sunnyvale, CA) according to the recommendations of the manufacturer and washed in 0.2 × SSC, 0.2% SDS twice for 1 hr at 68°C. Probes were labeled by the random prime method as suggested by the kit manufacturer (Amersham International). The gene-specific probe for *knotted1* (*kn1*) is H2, which was derived from a HindIII fragment from the third intron of the gene (Veit et al., 1990). The homeobox-specific probe was obtained by polymerase chain reaction amplification of the homeobox region of the *kn1* cDNA using the primers E35 and E40.

Genomic and cDNA Libraries

Genomic and cDNA libraries were screened with the homeobox-specific probe at reduced stringency under conditions similar to those described above for low-stringency DNA gel blot hybridizations. The genomic libraries were prepared from size-selected SacI fragments from *Kn1-N2* DNA in λ ZAPII according to the protocol supplied by Stratagene and from DNA partially digested with Sau3A in EMBL3 according to the protocols supplied by Stratagene. We screened cDNA libraries made from poly(A)$^+$-enriched mRNA from whole seedlings (provided by A. Barkan, University of Oregon, Eugene, OR) and from poly(A)$^+$-enriched mRNA isolated from ear primordia (Schmidt et al., 1993).

RNA Isolation and Blot Hybridization

Total RNA from various maize tissues was prepared as described by Smith et al. (1992). Poly(A)$^+$ enrichment was performed using the Poly Attract kit from Promega. Ten micrograms of total RNA or 1 μg of poly(A)$^+$-enriched RNA per lane was glyoxalated, separated on an agarose gel, transferred to Magna nylon transfer membranes, and hybridized as previously described (Smith et al., 1992). Blots were stripped and reprobed as many as five times. Autoradiograms were exposed for different amounts of time. The clones used to probe RNA gel blots were complete or partial maize cDNAs, except for KNOX4, and are as follows; KN1, a 1.6-kb cDNA clone; KNOX8, a 0.6-kb cDNA clone; RS1, an 0.8-kb cDNA clone; KNOX3, a 0.9-kb cDNA clone; KNOX4, a polymerase chain reaction product derived from a genomic clone that includes the ELK region, homeobox, a 127-bp intron, and 70 bp of the 3′ end of the gene; KNOX1, a 1.0-kb cDNA clone; KNOX2, a 1.0-kb cDNA clone; KNOX6, a 0.7-kb cDNA clone; and UBI1, a 0.65-kb PstI-SacI subclone of the maize ubiquitin cDNA clone (Christensen and Quail, 1989).

The specificity of hybridization of the KNOX probes used on RNA gel blots was checked on slot blots. Each KNOX clone was transcribed into RNA using T3 RNA polymerase and then treated with DNase to remove the DNA template (Sambrook et al., 1989). Three different quantities (100, 1.0, and 0.01 ng) of each RNA were loaded in a MinifoldII slot blotting device (Schleicher & Schuell) and transferred to a nylon transfer membrane according to Sambrook et al. (1989). The RNA and membrane were UV cross-linked and then baked for 1 hr at 80°C. The hybridization conditions were identical to those used for RNA gel blots.

Mapping Genes on Maize Recombinant Inbred Lines

Genomic DNA was isolated from leaves of two maize recombinant inbred (RI) families (CO159 × Tx303 and T232 × CM37) grown from seed provided by B. Burr (Brookhaven National Laboratory, Upton, NY). Survey gel blots were prepared with 7 to 10 μg of DNA per lane from each of the inbred parents restricted with various endonucleases. The blots were probed with gene-specific pieces derived from either the 3′ end of cDNA clones or the intron dividing the homeobox of genomic clones to identify appropriate polymorphisms. Combinations of RI family, restriction endonuclease, and probe that yielded useful polymorphisms were then used in the preparation of DNA gel blots containing the 41 or 48 RI individuals. The allelic distribution was then scored and sent to B. Burr for comparison with the distribution of all previously mapped loci (Burr et al., 1988). The linkage information returned was used to position the genes on the maize genome map, portions of which are shown in Figure 6.

Oligonucleotide Synthesis

Oligonucleotides were synthesized on a DNA synthesizer (model 380B; Applied Biosystems, Foster City, CA). Primers for amplification of the *kn1* homeobox are as follows: E35, 5′-CTCGCTCAAGCAAGAACTGTC-3′; E40, 5′-TCCATCATCAGGTGGTGCAT-3′. Degenerate oligonucleotides for sequencing are as follows: E44, 5′-CARATKAAYAAYTGGTTYATY-AAYCARMG-3′; E61, 5′-CAYNNNAAGTGGCCNTAYCC-3′; E62, 5′-CTTNCKYTGRTTRATRAACCA-3′, where R = A or G, Y = C or T, K = G or T, M = A or C, and N = A or G or C or T.

DNA Sequencing and Analysis

Double-stranded DNA sequencing was performed using the Sequenase Version 2 kit (U.S. Biochemical Corp.) according to the manufacturer's protocols. DNA and putative protein sequences were analyzed using the PCGENE program and the IG Molecular Biology Software System Release 5.4 (IntelliGenetics, Mountain View, CA). The dendrogram in Figure 6 was generated using the CLUSTAL program in PCGENE.

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Sequence analysis and expression patterns divide the maize knotted1-like homeobox genes into two classes.
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