

# A *knotted1*-like Homeobox Gene in Arabidopsis Is Expressed in the Vegetative Meristem and Dramatically Alters Leaf Morphology When Overexpressed in Transgenic Plants

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The homeobox gene *knotted1* (*kn1*) was first isolated by transposon tagging a dominant leaf mutant in maize. Related maize genes, isolated by virtue of sequence conservation within the homeobox, fall into two classes based on sequence similarity and expression patterns. Here, we report the characterization of two genes, *KNAT1* and *KNAT2* (for *knotted*-like from *Arabidopsis thaliana*) that were cloned from Arabidopsis using the *kn1* homeobox as a heterologous probe. The homeodomains of *KNAT1* and *KNAT2* are very similar to the homeodomains of proteins encoded by class 1 maize genes, ranging from 78 to 95% amino acid identity. Overall, the deduced *KNAT1* and *KNAT2* proteins share amino acid identities of 53 and 40%, respectively, with the *KN1* protein. Intron positions are also fairly well conserved among *KNAT1*, *KNAT2*, and *kn1*. Based on in situ hybridization analysis, the expression pattern of *KNAT1* during vegetative development is similar to that of class 1 maize genes. In the shoot apex, *KNAT1* transcript is localized primarily to the shoot apical meristem; down-regulation of expression occurs as leaf primordia are initiated. In contrast to the expression of class 1 maize genes in floral and inflorescence meristems, the expression of *KNAT1* in the shoot meristem decreases during the floral transition and is restricted to the cortex of the inflorescence stem. Transgenic Arabidopsis plants carrying the *KNAT1* cDNA and the *kn1* cDNA fused to the cauliflower mosaic virus 35S promoter were generated. Misexpression of *KNAT1* and *kn1* resulted in highly abnormal leaf morphology that included severely lobed leaves. The expression pattern of *KNAT1* in the shoot meristem combined with the results of transgenic overexpression experiments supports the hypothesis that class 1 *kn1*-like genes play a role in morphogenesis.

## INTRODUCTION

Development of the shoot portion of plants originates from a distinct region of the shoot apex, termed the shoot apical meristem. The shoot meristem forms during embryogenesis and functions throughout the vegetative and reproductive phases of plant development to repetitively generate lateral organs, such as leaves or flowers, and stem tissues. In addition to initiating new tissues and organs, the shoot meristem must maintain or replenish itself (Steeves and Sussex, 1989). In the shoot apical meristem of most angiosperms, cytological variations have led to the description of three major regions or zones. The central zone consists of slowly dividing initials that serve as a source of cells for other regions of the meristem. The peripheral zone surrounds the central zone and consists of more rapidly dividing cells undergoing organogenesis; lateral

organs such as leaf primordia arise in a precise, genetically determined pattern (phyllotaxy) within this zone. Finally, the rib zone forms a transition between the apical meristem and the rest of the shoot apex and contributes tissues to the pith or central portion of the stem (Wardlaw, 1957; Medford, 1992). Despite extensive knowledge of the complex structural organization of the shoot apical meristem, very little is known about the molecular mechanisms responsible for meristem initiation and function.

Fate mapping experiments have shown that cell position is more important than cell lineage in determining the final pattern of differentiated tissues within the shoot (reviewed in Poethig, 1989; Irish, 1991). How do cells in the shoot meristem acquire positional information and then differentiate accordingly? In animals, genetic analysis has shown that cells receive information from regulatory molecules whose expression patterns are established early in embryo development. The products of homeobox genes are one such class of regulatory molecules (Gehring, 1987; McGinnis and Krumlauf, 1992). The homeobox, first identified in *Drosophila* homeotic genes, and

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subsequently in other invertebrate, vertebrate, fungal, and plant genes, encodes the evolutionarily conserved DNA binding homeodomain (Gehring et al., 1990). High-resolution structural studies of homeodomain-DNA complexes have shown that the homeodomain is composed of three major  $\alpha$ -helical regions; residues in the highly conserved third helix and in the N-terminal arm of the homeodomain play crucial roles in sequence-specific recognition of DNA (Qian et al., 1989; Kissinger et al., 1990; Otting et al., 1990; Wolberger et al., 1991). Homeodomain proteins are thought to act as transcriptional regulators that control the expression of specific target genes.

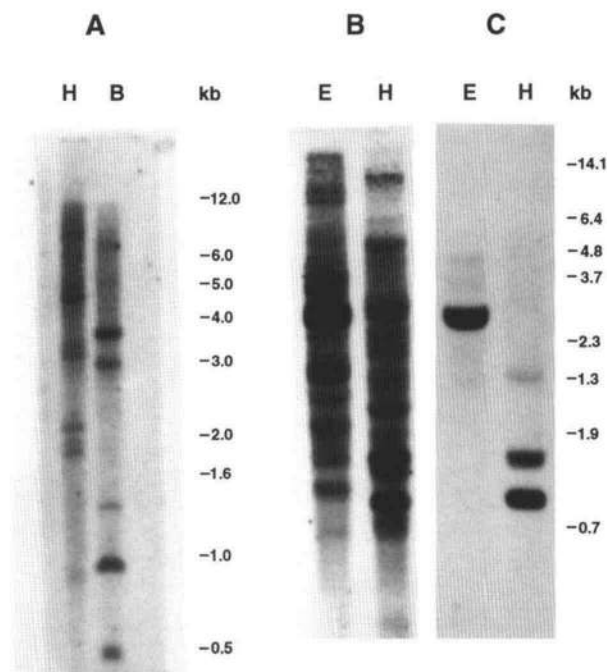
Amino acid sequence similarities within the homeodomain and flanking regions have been used to subdivide the large number of known homeodomain proteins into related classes or families, such as the Antennapedia (*Antp*), engrailed (*en*), and POU classes (Scott et al., 1989). Four novel classes of homeobox genes have been identified in several plant species, including maize (Vollbrecht et al., 1991; Bellman and Werr, 1992), rice (Matsuoka et al., 1993), soybean (Ma et al., 1994), parsley (Korfhage et al., 1994), and Arabidopsis (Ruberti et al., 1991; Mattson et al., 1992; Schena and Davis, 1992; Schena et al., 1993; Schindler et al., 1993; Korfhage et al., 1994; Rerie et al., 1994). Although there are dramatic differences in the developmental processes used by plants compared with animals, the discovery of plant homeobox genes suggests that some of the fundamental regulatory mechanisms that control development have been conserved between these two distinct groups of organisms.

Characterization of the maize homeobox gene, *knotted1* (*kn1*), provided the first evidence that plant homeobox genes may function in specifying cell fate. The *kn1* locus is defined by a series of dominant neomorphic mutations that disrupt leaf development, leading to the formation of finger-like outgrowths, or knots, on the leaf blade (Freeling and Hake, 1985; Vollbrecht et al., 1991; Hake, 1992). In *Kn1* mutants, ectopic expression of the *kn1* gene product in developing leaves has been correlated with the mutant phenotype. Although the role of the wild-type *kn1* gene product is unknown, its expression pattern during vegetative and floral development in mutant and wild-type plants suggests that *kn1* participates in the switch from indeterminate to determinate cell fates (Smith et al., 1992; Jackson et al., 1994). Overexpression of *kn1* in transgenic tobacco plants causes leaf cells to take on alternate cell fates. The transgenic tobacco phenotypes range from abnormal leaf shapes to the formation of ectopic shoots on leaf surfaces (Sinha et al., 1993).

The *kn1* homeobox sequence was used to isolate a family of maize homeobox genes that have been named *knox*, for *knotted*-like homeobox (Kerstetter et al., 1994). In addition to encoding homeodomains similar to those encoded by *kn1*, the *knox* gene products share a novel motif consisting of 24 amino acids adjacent to the N-terminal side of the homeodomain (Vollbrecht et al., 1991, 1993). The expression patterns of *kn1* and three *knox* genes have been examined during vegetative development. In the shoot apex, the expression profiles of these

maize homeobox genes appear to predict or define morphogenetic events such as leaf initiation (Jackson et al., 1994).

We are interested in elucidating the role of *kn1* and related homeobox genes in plant development. Toward this goal, we have used the homeobox sequence of *kn1* to isolate a family of related homeobox genes in Arabidopsis. The existence of *knox* homologs in Arabidopsis will permit further molecular and genetic analyses of this class of homeobox genes. In addition, sequence comparisons across species should help determine important functional domains within these genes. Finally, based on the expression pattern of *kn1* and related genes in the shoot apex of maize, we anticipate that homologous genes will be useful markers for meristem organization in Arabidopsis. In this paper, we describe the isolation and characterization of two genes, *KNAT1* and *KNAT2* (for *knotted*-like from *Arabidopsis thaliana*) encoding KN1-like homeodomain proteins in Arabidopsis.



**Figure 1.** Arabidopsis Genomic DNA Gel Blot Analysis.

(A) Hybridization with the *kn1* homeobox. Genomic DNA from the Columbia ecotype digested with either HindIII (H) or BglIII (B) was hybridized with the *kn1* sequence encoding the ELK and homeodomain under low-stringency conditions. DNA length markers are given at right in kilobases.

(B) Hybridization with *KNAT1* cDNA. Genomic DNA from the Columbia ecotype digested with either EcoRI (E) or HindIII (H) was hybridized with the full-length *KNAT1* cDNA at low stringency.

(C) Hybridization with the *KNAT1* homeobox. The blot in (B) was stripped and reprobed with the *KNAT1* homeobox sequence at low stringency. HindIII cuts within the *KNAT1* homeobox, generating the two bands between 1.9 and 0.7 kb. Molecular length markers are given at right in kilobases.

TTATTTTCTTCTTCTTCCATGTCACCTCTCCCTGACGAATTCATATACCTAGTTCGTTTTTT  
 CTTCCTCAAATATATCTTTTTCAATTTATTTGGTTTTCTTTGGTGCACCTTCACCTCA  
 CAAAATTTTCTCTTTTTTTATATTAATTTGAGTTAGGCCTTTTTGATTCATAGATGA  
**GTCGCTAGTCTGCTGGATTG**ATGTGGTTATAGTCTTACAGAGACCTTTGATTGAAATA  
 AGAACAAAAGCAAGAATACATACCTCTTCATCTTACACCCCATCTTTTTTATTTTTCT  
 AGGGTTTTATTTTTTTAATTTATTTTTTCTTTGATTTTTATATCTCTCTCTCTCT  
 CAAATCTTTACTCATCTGGGTATGGAAGAATACCAGCATGCAACAGCACCACTCTC  
 M E E Y Q H D N S S T P Q 13

AGAGTAAGTTCTGTACTCTCCAATCTCTCTTCCAACAAAACGATAACACAAGTAT  
 R V S F L Y S P I S S S N K N D N T S D 33

ACCAACAACAACAACAATAAATAGTAGCAATATGGTCTGGTTACAATAATACT  
 T N N N N N N N N S S N Y G P G Y N N T 53

AACAACAACAATCATCACCACCAACACATGTGTTCACATATGAGCTCTCTCTCCCT  
 N N N N H H H Q H M L F P H M S S L L P 73

CAAACAACCGAGAATTGCTCCGATCTGATCATGATCAACCAACAACAACAACCCA  
 Q T T D N C F R S D H D Q P N N N N N P 93

TCTGTTAAATCTGAAGCTAGCTCTCAAGAATCAATCATTACTCCATGTTAATGAGAGCC  
 S V K S E A S S S R I N H Y S M L M R A 113

ATCCACAATCTCAAGAAGCTAACAACAACAACAATGACAACCGTAAGCGATGTTGAAGCC  
 I H N T Q E A N N N N N D N V S D V E A 133

ATGAAGGCTAAAATCAATTGCTCATCTCACTACTCTACCTCCTACAAGCTTACTGGAC  
 M K A K I I A H P H Y S T L L Q A Y L D 153

TCGCAAAAGATTGGAGCTCCACCTGATGTGGTTGATAGAATTCGGCGGCACGGCAAGAC  
 C Q K I G A P P D V V D R I T A A R Q D 173

TTTGAGGCTCGACACAGCGGTCAACACCGTCTGTCTCTGCCTCCTTAGAGACCGGAG  
 F E A R Q Q R S T P S V S A S S R D P E 193

TTAGATCAATTCATGGAAGCATCTGTGACATGTTGGTTAAATATCTGAGGAGCTAACCA  
 L D Q F M E A Y C D M L V K Y R E E L T 213

AGGCCCATTCAGGAAGCAATGAGTTTATACGTCGATTGAATCTCAGCTTAGCATGTTG  
 R P I Q E A M E F I R R I E S Q L S R M L 233

TGTCAGAGTCCCATTCACATCTCAACAATCTCTGATGGGAAGTGACAATATGGGATCA  
 C Q S P I H I L N N P D G K S D N M G S 253

TCAGACGAAGAACAAGAGAATAACAGCGGGAGGGAACAAGAAATACCGGAAATAGACCCG  
 S D E E Q E N N S G G E T E L P E I D P 273

AGGCCGAAGATCGGGAATCAAGAACCTTTGCTGAAGAAGTATAGTGGATACTTAAGC  
 R A E D R E L K N H L L K K Y S G Y L S 293

AGTTTGAAGCAAGAATCTCAAGAAGAAAAGAAAGTTAACTTCTTAAAGACACGG  
 S L K Q E L S K R K K K G K L P K E A R 313

CAGAAGCTTCTCAGTGGTGGGAGTTGCATTACAAGTGGCCATATCTTCTGAGTCAGAG  
 Q H L L T W F E L H Y K W P Y P S E S E 333

AAGGTAGCGTTGGCGGAATCAACGGGGTTAGATCAGAAACAATCAACAATTTGTTTCA  
 K V A L A E S T G L D Q K Q I N N W F I 353

AACCAAGAAAGCGTCACTGGAACCATCTGAAGACATGCAGTTTATGTTGATGGATGGT  
 N Q R K R H W K P S E D M Q F M V M D G 373

CTGCAGCACCCGACCCAGCAGCTCTGTACATGGATGGTCAATACATGGGTGATGGACCT  
 L Q H P H H A A L Y M D G H Y M G D G P 393

TATCGTCTCGGTCCATAAGACATCCAAAGCTTTAGCCACATAATAACAACCTCTCGTTG  
 Y R L G P 398

CTTTCTTGTTCACAACTCATGTTTTGAATCCCCCTACATCAGTTTGTCTACTTATAGCTTTC  
 TTGTTTTACCGCTTTTGTAAATGTCTTATGTCTGACGGGGAGTTTGTAGACTTCTAGTC  
 AGAAATATCCCTTCATTTTATTTCTCTTTTTTCTCGGTGTATTTTGTTTTTTGTTTTTCT  
 CACTAATGTTTTTATTTAATAATGTGTGAAAGGAAAATGCTATTCTGAAA

Figure 2. Nucleotide and Deduced Amino Acid Sequence of *KNAT1*.

The open triangle at the 5' end indicates the 5' end of the longest cDNA; the sequence upstream was derived from genomic clones. Hooked arrows indicate putative transcription start sites. Short open reading frames upstream of the translation start site are indicated by boldface type. The asparagine- and histidine-rich regions are double underlined. Sites for introns 1 to 4 are marked by filled triangles. The

RESULTS

Isolation of *kn1*-like Genes from Arabidopsis

*kn1* and related *knox* genes in maize encode a highly conserved block of 88 amino acids that begins with the ELK region (24 amino acids; named for invariant series of Glu, Leu, and Lys amino acids) and extends through the adjacent homeodomain (64 amino acids; Vollbrecht et al., 1993; Kerstetter et al., 1994). When an Arabidopsis genomic DNA gel blot was hybridized at reduced stringency with the *kn1* sequence encoding this conserved extended homeodomain region, approximately six bands of different intensities were detected, as shown in Figure 1A, suggesting the presence of a family of *kn1*-like genes. Using the same sequence as a probe, several different Arabidopsis cDNA libraries, made from either seedling, adult vegetative, or floral tissues, were screened. Independent cDNA clones were isolated and sequenced using degenerate oligonucleotide primers derived from the third helix of the *kn1* homeobox. In this study, we have focused on the characterization of cDNAs and the corresponding genomic clones representing two different genes, *KNAT1* and *KNAT2*.

The longest *KNAT1* cDNA sequence, shown in Figure 2, contains a 1197-bp open reading frame encoding a polypeptide of 398 amino acids. The open reading frame present in the longest *KNAT2* cDNA, shown in Figure 3, encodes a putative polypeptide of 310 amino acids. The predicted amino acid sequence of *KNAT1* is compared with *KN1* in Figure 4A. Overall, the two sequences share 53% amino acid identity. The highest identity is found within the 88 amino acids of the extended homeodomain region (the ELK region plus the homeodomain) located near the C terminus: the ELK regions are 87.5% identical, and the homeodomains are 89% identical. The homeodomain of *KNAT2* is 80% identical to both *KNAT1* and *KN1* (Figure 4A). When the entire amino acid sequences are compared, the degree of identity between *KNAT2* and either *KNAT1* or *KN1* drops to ~40%. The homeodomains of *KNAT1* and *KNAT2* contain the four invariant amino acids (WF-N-R), as well as identical residues or similar substitutions for six of the eight highly conserved amino acids of the animal homeodomain consensus sequence. Within the third helix (the "recognition helix") of the homeodomain, 13 consecutive amino acids are completely conserved in all *KN1*-like proteins, including both *KNAT1* and *KNAT2*.

The *KNAT1*, *KNAT2*, and *KN1* amino acid sequences share several interesting features in addition to the conserved

suggested acidic region is marked by asterisks. The homeodomain is indicated by a filled bar, and the putative nuclear localization sequence is marked by a dashed line. A putative polyadenylation signal is indicated by single underlining. Numerals at right indicate amino acid positions. Sequence data have been submitted to GenBank as accession numbers U14174 and U14175 for *KNAT1* and *KNAT2*, respectively.

CACAAGGCTAGGGATCGAAGAAGCGCGATCACTGATCGTATCTCACTACGATCACATTA  
 ATGGATAGAAATGTGTGGTTCCCGCTCGACGGAAGACTATTCGGAGAAAGCGACTTGTATG  
 M D R M C G F R S T E D Y S E K A T L M 20  
 ATGCCGTCGCGATTATCAGTCTTTGATTTGTTCAACCAACCGGAGACAATCAAGACTGTTT  
 M P S D Y Q S L I C S T T G D N Q R L F 40  
 GGATCCGACGAACCTCGCTACCGCTTTGCTCTCGAGTTCGCGTATTCGAAAGCT  
 G S D E L A T A L S S E L L P R I R K A 60  
 GAGGATAATTTCTCTCTAGTGTATCAAAATCAAAATCGCTTCTCATCTTTGTATCCT  
 E D N F S L S V I K S K I A S H P L Y P 80  
 CGCTTACTCCAAACCTACATCGATTGCCAAAGGTGGAGCGCCTATGGAATAGCGTGT  
 R L L Q T Y I D C K V M E T Y C D I L V 100  
 ATATTGGAAGAGATTACGCGAGAGAACCATGTGTACAAGAGAGATGTGCTCCATTATCT  
 I L E E I Q R E N H V Y K R D V A P L S 120  
 TGCTTTGGAGTGATCCTGAGCTTGATGAATTCATGGAACACTCTGTATATATGGTT  
 C F G A D P E L D E F M E T Y C D I L V 140  
 AAATACAAAACCGATCTCGGAGGCCCTCGACGAGGCTACAACCTTCATAAACAAGATT  
 K Y K T D L A R P F D E A T T F I N K I 160  
 GAAATGCAGCTTCAGAACCTGCACTGCTCCAGCGTCTGCTACAGCTCTTTACGATGAT  
 E M Q L Q N I L C T G P A S A T A L S D D 180  
 GGTGCGGTTTCACTGACGAGGAACAGAGAAGATGATGACATAGCAGCGGATGACAGC  
 G A V S S D E E L R E D D D I A A D D S 200  
 CACAAGAAGCAATGACCGCATCTGAAGGACAGCTACTACGCAATTTGATAGCCAT  
 Q Q R S N D R D L K D Q L L R K F G S H 220  
 ATCAGTTCAATGAAACTCGAGTCTCTAAAAAGAAGAAGGGAAGCTACCAAGAGAA  
 I S S L K L E F S K K K K K G K L P R E 240  
 GCAAGACAAGCGTTGCTGATTTGGTGAATGTTCAATAAATGGCCTTACCCTACTGAA  
 A R Q A L L D W W N V H N K W P Y P T E 260  
 GGCACAAAATATCTCTGCTGGAAGAAACAGGTTTGGATCAAAAACAAATCAACAATTTGG  
 G D K I S L A E E T G L D Q K Q I N N W 280  
 TTTATAACCAAGGAAACGCAATTTGGAAGCCTTCGGAGAACATGCGGTTTGATATGATG  
 F I N Q R K R H W K P S E N M P F D M M 300  
 GACGATTTAATGAAACATCTTTTACCGAGTAATGAAAGAGAGACATGAAATTTGTGCAT  
 D D S N E T F F T E 310  
 TGTATAATTTTACACTGTTTTCACAGAAAAGAAAACAGTAAAAGCTTTTGGTAAATG  
 GGCATCATCGCAATGAAATGGAACCCGTTAGCCAAAACCGTCAAGGCGCTAACCGAGACA  
 TTGTATTGGAATAGTGGCAATATTAATGTCACAAATCTTCCAATCATCAAATTTGATAGA  
 TTCTTATTGATTTGAACTTACTTAGATAGCTGATGTGTCAACTAAATAATTTATTTT  
 CATTATCCATTCGGGTAGA

**Figure 3.** Nucleotide and Deduced Amino Acid Sequence of *KNAT2*. Symbols used in this figure are as given in Figure 2.

homeodomain and ELK region. All three polypeptides contain a putative nuclear-targeting sequence located in the basic region preceding the first helix of the homeodomain (Figures 2 and 3; Raikhel, 1992; Varagona et al., 1992). Previous studies have shown that the KN1 protein is localized to maize nuclei (Smith et al., 1992). Immediately upstream of the ELK regions of *KNAT1*, *KNAT2*, and *KN1*, there is a cluster of negatively charged amino acids (Figures 2 and 3). Similar acidic domains are found in other classes of plant homeodomain proteins (Carabelli et al., 1993; Ma et al., 1994; Rerie et al., 1994). Acidic regions have been postulated to function as transcription activation domains (Ptashne and Gann, 1990; Frankel and Kim, 1991). Near the N terminus of *KNAT1*, there are four short regions rich in asparagine (Figure 2). A polyhistidine block is present near the N terminus of *KN1* (Vollbrecht et al., 1991);

**A**

*KNAT1* MPEYQHDNSSTPQRVSFLYSPISSSNKNDNTSDTNMNNMNSNYGPGY  
*KN1* MEEITQHPGVGA-----SSHGICH  
*KNAT2* MD-----RMC-----GP

*KNAT1* NNTNNNHHHQH---MLPPHMSSLLPOTDNCFRSDHQPNMNNPVSKE  
*KN1* GQHHHHHHHPWASSLSAVVALPQPQPSAGLPLTLNTVAATGNSGGSG  
*KNAT2* RSTED-----YSEKAILMPSDYQSL---ICSTTGDNQRLFGS

*KNAT1* EASSSRINHYSMIRALHNTCEANNNNNDVSDVEAMKAKITLHPHYSTL  
*KN1* NPVL-QLANGGGLLACVKAMPEPSSSSP-YAGDVEAIKAKIISHPHYVSL  
*KNAT2* DELATALS--SELLPRIKARDNFS-----LSVKKSKIAHSHLYPRL

*KNAT1* LQAYLDCQRIIGAPPLVVDRIIFAARDFEARQORSTPSVSAASSRDPELDQF  
*KN1* LAYLE NKVGAPEVRSARLTETAQVEARQRTALGCLAAT-EPELDQF  
*KNAT2* LTYIDCQKVGAPMELACILLETQREWHYKRDVAP-LSCFGADPELDFE

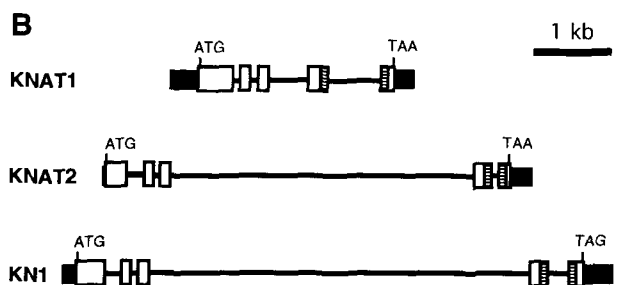
*KNAT1* MEAYCDMLVKYREELTRPQEMEFIRIEGQLSMLCQSEPIHILNPPGDK  
*KN1* MEAYTEMLVKREELTRPQEMEFIRIEGQLSMLCQSEPIHILNPPGDK  
*KNAT2* MEAYCDMLVKYKTDLARPDEATTFENKTEMOLNLCGPA--SATAALS

*KNAT1* SDNMGSSDEEIQENNSCGGETELPEHDPRAEDRELKNIHLKKYSGYLSSLKQ  
*KN1* NILSSGSSDEEIQENNSCGGETELPEHDPRAEDRELKNIHLKKYSGYLSSLKQ  
*KNAT2* DSAVSSDEELREDDDIAD--DSQQRNSDRFLKQDLRLKFGSHSSLKL

*KNAT1* ELSKKKKKGLPKEARQKLLWVWVHYKWPYPSSEKVALAESTGLDQKQ  
*KN1* ELSKKKKKGLPKEARQKLLWVWVHYKWPYPSSEKVALAESTGLDQKQ  
*KNAT2* ELSKKKKKGLPKEARQKLLWVWVHYKWPYPSSEKVALAESTGLDQKQ

*KNAT1* INNWFINQRRKRWKPSSEEMFVMDGHLQHPHHAALYMDGHYMGDGP-YRL  
*KN1* INNWFINQRRKRWKPSSEEMHLLMMDGYHTN--AFYMDGHYINDGGLYRL  
*KNAT2* INNWFINQRRKRWKPSSEEMFVMDMD--SNETFPTE-----

*KNAT1* GP 398  
*KN1* G 359  
*KNAT2* -- 310



**Figure 4.** Comparison between the Protein Sequences and Genomic Organization of *KNAT1*, *KNAT2*, and *KN1*.

(A) Comparison of *KNAT1*, *KNAT2*, and *KN1* protein sequences. The deduced amino acid sequences of *KNAT1* and *KNAT2* are compared with the previously published sequence of *KN1* (Vollbrecht et al., 1991). Gaps, indicated by hyphens, were introduced to facilitate alignment. Identical residues are boxed in black. The ELK region is underlined with a hatched bar, and the homeodomain is underlined with a solid bar. Conserved intron positions (within 0 to 2 bp) are indicated by filled triangles. The intron 3 position is variable and is indicated with an open triangle containing a number: (1) *KNAT1*, (2) *KNAT2*, (3) *kn1*.  
 (B) Intron-exon structure of *KNAT1* and *KNAT2* compared with *kn1*. Filled boxes indicate untranslated regions. Exons and introns are indicated by open boxes and thick lines, respectively. Homeobox sequences are shown by striped boxes.

the *KNAT1* amino acid sequence contains a much shorter stretch of histidines (Figure 2). Amino acid homopolymers are found in the protein-coding regions of many genes that have important developmental roles (Laughon et al., 1985; Wharton et al., 1985). Asparagine-rich sequences occur in several transcription factors of various types, including homeodomain proteins, although their function is not known (Smoller et al., 1990; Mevel-Ninio et al., 1991). Recent evidence suggests that polyglutamine and polyproline stretches may be involved in transcriptional activation (Gerber et al., 1994).

In contrast to *kn1*, there are two short open reading frames in the 5' untranslated sequence of *KNAT1* (Figure 2). Similar short open reading frames have been identified in the leader sequences of *Athb-1* and *Athb-2*, both members of the homeodomain zipper family of genes in Arabidopsis (Ruberti et al., 1991; Carabelli et al., 1993) and *sbh1*, a *kn1*-like homeobox gene in soybean (Ma et al., 1994). The function of such leader peptides is not clear, but they may play a role in translational regulation, as demonstrated for GCN4 expression in yeast (Hinnebusch, 1988) and *opaque2* in maize (Lohmer et al., 1993).

Genomic clones corresponding to *KNAT1* and *KNAT2* were isolated from a  $\lambda$  DNA library, and exons were defined by comparing genomic and cDNA sequences. The genomic organization of both genes is shown in Figure 4B. All intron-exon junctions conform to consensus splice sites for plant introns (Brown, 1986). Although intron sizes differ greatly (Figure 4B), the positions of introns 1, 2, and 4 in both *KNAT* genes are almost identical to those in *kn1* (Vollbrecht et al., 1991). These introns are in regions well conserved among *KNAT1*, *KNAT2*, and *KN1* (Figure 4A). Intron 3 is in a less conserved region, and the intron position is more variable. The homeoboxes of *KNAT1* and *KNAT2* are interrupted by the fourth intron, which is located between helix 1 and helix 2 of the homeodomain. The position of this intron is conserved in *kn1* and all of the isolated *knox* genes (Kerstetter et al., 1994).

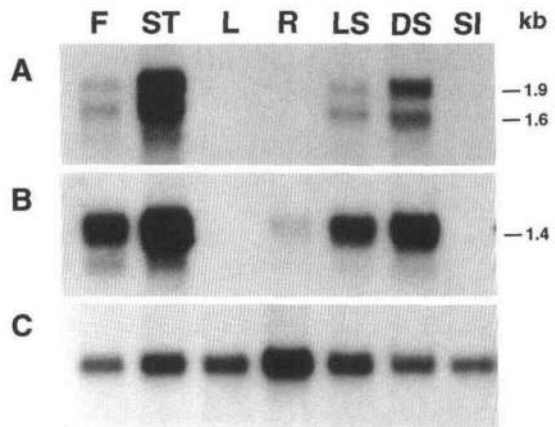
High-stringency hybridization of a 5' noncoding portion of *KNAT1* or *KNAT2* to a gel blot containing Arabidopsis DNA digested with several enzymes detected one or two bands characteristic of single-copy genes (data not shown). Hybridization of the entire *KNAT1* cDNA under low-stringency conditions detected a large number of bands (Figure 1B). This result is presumably due to the repetitive nature of the 5' coding region of *KNAT1*, because the homeobox sequence alone detected many fewer bands with low-stringency hybridization (Figure 1C). Based on the results of DNA gel blot hybridizations (Figure 1A) and characterization of clones identified in cDNA library screens (C. Lincoln and K. Serikawa, unpublished data), *KNAT1* and *KNAT2* appear to be members of a relatively small family of *kn1*-like genes in Arabidopsis.

The chromosomal locations of *KNAT1* and *KNAT2* were identified by determining the segregation of restriction fragment length polymorphisms, specific for each gene, among recombinant inbred lines. The *KNAT1* gene maps to chromosome 5 at an approximately equal distance from markers m291 and r355.2 (Reiter et al., 1992). The *KNAT2* gene maps to

chromosome 1 between markers g4552 and m532 (Lister and Dean, 1993). At the time of publication, no mutant phenotypes had been mapped to the position of either *KNAT1* or *KNAT2*.

**Analysis of *KNAT* Gene Expression**

Expression of *KNAT1* and *KNAT2* was examined using RNA blot analysis of poly(A)<sup>+</sup>-enriched RNA isolated from various Arabidopsis tissues. To avoid cross-hybridization of probes to related mRNAs, gene-specific probes were used and hybridizations were performed at high stringency. Figure 5A shows that two *KNAT1* transcripts are detected in mRNAs isolated from flowers, inflorescence stems, and light- and dark-grown seedlings. In these tissues, the abundance of the two transcripts relative to one another did not vary significantly. Expression of both the large (~1.9 kb) and small (~1.6 kb) transcripts was highest in floral stem tissue. The level of *KNAT1* expression was higher in dark-grown seedlings compared with light-grown seedlings. In situ hybridization experiments (see following discussion) have shown that the *KNAT1* transcript is localized primarily to the shoot apex of young, light-grown seedlings. The tissue localization of expression in dark-grown seedlings was not determined. As shown in Figure 5A, *KNAT1* expression was not detected in leaves, roots, or siliques.



**Figure 5.** Analysis of *KNAT1* and *KNAT2* Expression in Different Organs of Arabidopsis.

(A) RNA gel blot analysis of *KNAT1* expression. RNA was isolated from flowers (F) of all stages, inflorescence stems (ST), rosette leaves (L), roots (R), light-grown seedlings (LS), dark-grown seedlings (DS), and siliques (SI) of all stages. Two micrograms of poly(A)<sup>+</sup> RNA was loaded per lane. The blot was hybridized with a 5'-specific probe of *KNAT1*. (B) RNA gel blot analysis of *KNAT2* expression. The blot in (A) was reprobbed with a 5'-specific probe of *KNAT2*.

(C) Control hybridization. The same blot was reprobbed with the constitutively expressed cytosolic cyclophilin gene *ROC1* (Lippuner et al., 1994) to show the relative quantity of RNA in each lane.

The approximate length of each transcript is shown at right in (A) and (B).

The estimated length of the longer *KNAT1* transcript identified in RNA blots closely agrees with the length of the longest *KNAT1* cDNA (1859 bp). The results of RNase protection experiments (data not shown) indicated that there are two potential transcription initiation sites close to the 5' end of the cDNA (Figure 2). The large and small *KNAT1* transcripts appear to correspond to the first and second transcription start sites identified in RNase protection experiments.

As shown in Figure 5B, the overall pattern of *KNAT2* expression is similar to that of *KNAT1*. The *KNAT2*-specific probe detected a single band (~1.4 kb) in flower, inflorescence stem, and light- and dark-grown seedlings, but, unlike *KNAT1*, the *KNAT2* probe also hybridized weakly to root RNA. No *KNAT2* expression was detected in leaves or siliques. Compared with *KNAT1*, the abundance of *KNAT2* transcript appears to be more uniform in flower, floral stem, and seedling tissues, with slightly higher expression in stem tissue. The results of RNase protection experiments (data not shown) indicated a potential transcription initiation site 27 bp 5' of the longest *KNAT2* cDNA (Figure 3).

### In Situ Localization of *KNAT1* Transcript

To determine the tissue localization of *KNAT1* transcript during vegetative and floral development, expression patterns were determined by in situ hybridization of digoxigenin-labeled RNA probes to tissue sections. The expression of *KNAT1* in vegetative tissues is shown in Figure 6. Control sections hybridized with sense RNA probes gave no signal above background staining (data not shown). High levels of *KNAT1* transcript were observed in the shoot apical meristem; however, the mRNA was not uniformly distributed throughout all layers or zones of the meristem. As shown in a nearly median longitudinal section (Figure 6A) and an adjacent median section (Figure 6B) of a 6-day-old shoot apex, the *KNAT1* message was localized primarily to the peripheral zone of the apical meristem. *KNAT1* expression was reduced or absent in the central zone of the meristem (Figure 6B). A low level of expression was detected at the bases of leaf primordia and in cells adjacent to the developing vasculature. No expression was observed in the hypocotyl (Figures 6A and 6B).

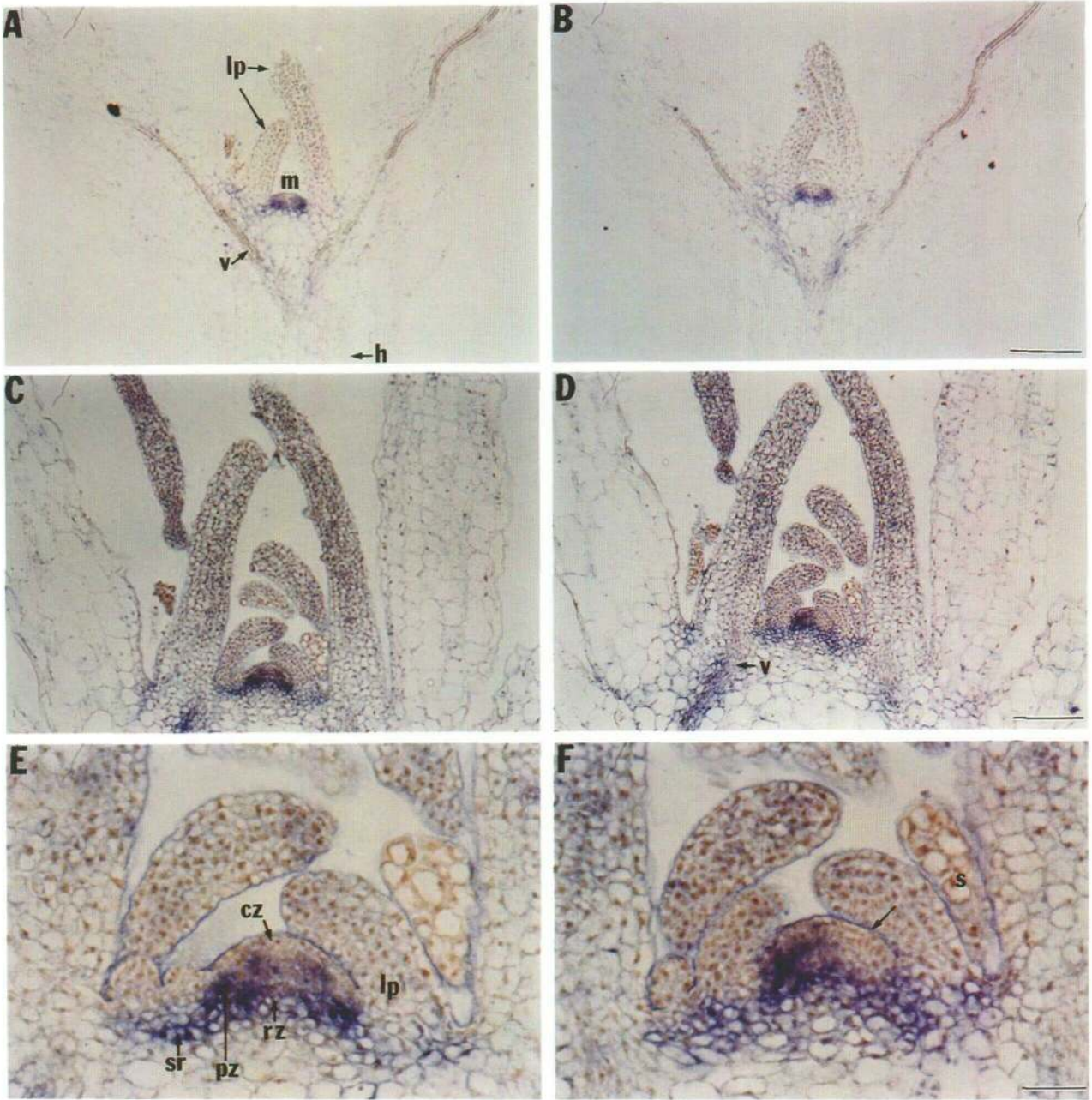
To examine further the expression pattern of *KNAT1* in the vegetative shoot apex, plants were grown under short-day (8-hr photoperiod) conditions to prolong vegetative growth and increase meristem size. Under long-day conditions (16- to 24-hr photoperiod), early flowering ecotypes, such as Columbia, produce five to eight vegetative rosette leaves and then bolt to form the inflorescence (Bowman, 1994). Although *Arabidopsis* (ecotype Columbia) grown under short-day conditions will eventually flower, the period of vegetative growth can be extended to 30 to 40 days in an 8-hr photoperiod compared with 8 to 10 days when plants are grown in a 16-hr photoperiod (Hempel and Feldman, 1994). A median longitudinal section through the shoot apex of a 23-day-old plant grown in 8 hr of light is shown in Figures 6C and 6E (higher magnification).

*KNAT1* expression was concentrated in the peripheral and rib zones of the apical meristem; however, high levels of transcript were also seen immediately below the meristem in the subapical region of the shoot apex, where shoot widening takes place (Wardlaw, 1957). Expression extended to the base of developing leaf primordia and was detected in cells adjacent to the differentiating vascular tissue of the stem (Figures 6C and 6D). The expression pattern shown in Figure 6C is similar to that observed in younger plants grown under long-day conditions (Figures 6A and 6B). There was no detectable message in young leaf primordia, older leaves, or stipules. Occasionally, faint, patchy staining was observed in leaves (Figures 6C and 6D). We have interpreted this staining as nonspecific because similar staining was observed with the sense control probe, and our RNA gel blot results as well as the majority of antisense in situ hybridizations did not detect any *KNAT1* RNA in leaves. Serial sections through the shoot apex of plants grown under short-day conditions revealed discrete areas or groups of cells on the flanks of the apical meristem where there was an absence of *KNAT1* expression (Figures 6E and 6F, higher magnification). This absence of *KNAT1* expression may correlate with leaf initiation sites. Control sections probed for 18S rRNA transcripts showed uniform signal throughout all tissues, indicating that the absence of *KNAT1* expression in some cells was not due to altered RNA retention or accessibility (data not shown).

The expression of *KNAT1* mRNA was also examined during the floral transition and the following stages of inflorescence and floral development. After floral induction in *Arabidopsis*, the shoot apical meristem enters the reproductive growth phase. We will use the term "inflorescence meristem" to refer to a shoot apical meristem that has undergone the floral transition. The primary inflorescence meristem of an indeterminate plant, such as *Arabidopsis*, gives rise to determinate floral meristems. Lateral, secondary inflorescences (paraclades), which are subtended by cauline (bract) leaves, arise as a result of a basipetal activation of buds (Hempel and Feldman, 1994). The growth pattern of the paraclades is essentially identical to the pattern exhibited by the primary inflorescence. After the development of several flowers and paraclades, rapid internode elongation (or bolting) occurs along the primary inflorescence axis (Bowman, 1994).

To investigate the pattern of *KNAT1* expression in the shoot apex during the transition to flowering, 16-hr photoperiods were used to synchronously induce flowering in plants grown for ~30 days in noninductive 8-hr photoperiods (see Methods). Shoot apices were harvested and fixed after different lengths of time following the start of the inductive photoperiod. As shown in Figure 7, the distribution of *KNAT1* transcript in the shoot apex is altered following floral induction. At hour 16 postinduction, *KNAT1* expression was still concentrated in the peripheral zone of the meristem; lower levels of mRNA were detected in the subapical region of the shoot apex, at the base of leaf primordia, and in cells adjacent to developing vascular tissue in the stem (Figure 7A). A late-transition-stage apex, characterized by expansion of the rib meristem and doming





**Figure 6.** *KNAT1* Expression in the Vegetative Shoot Apex.

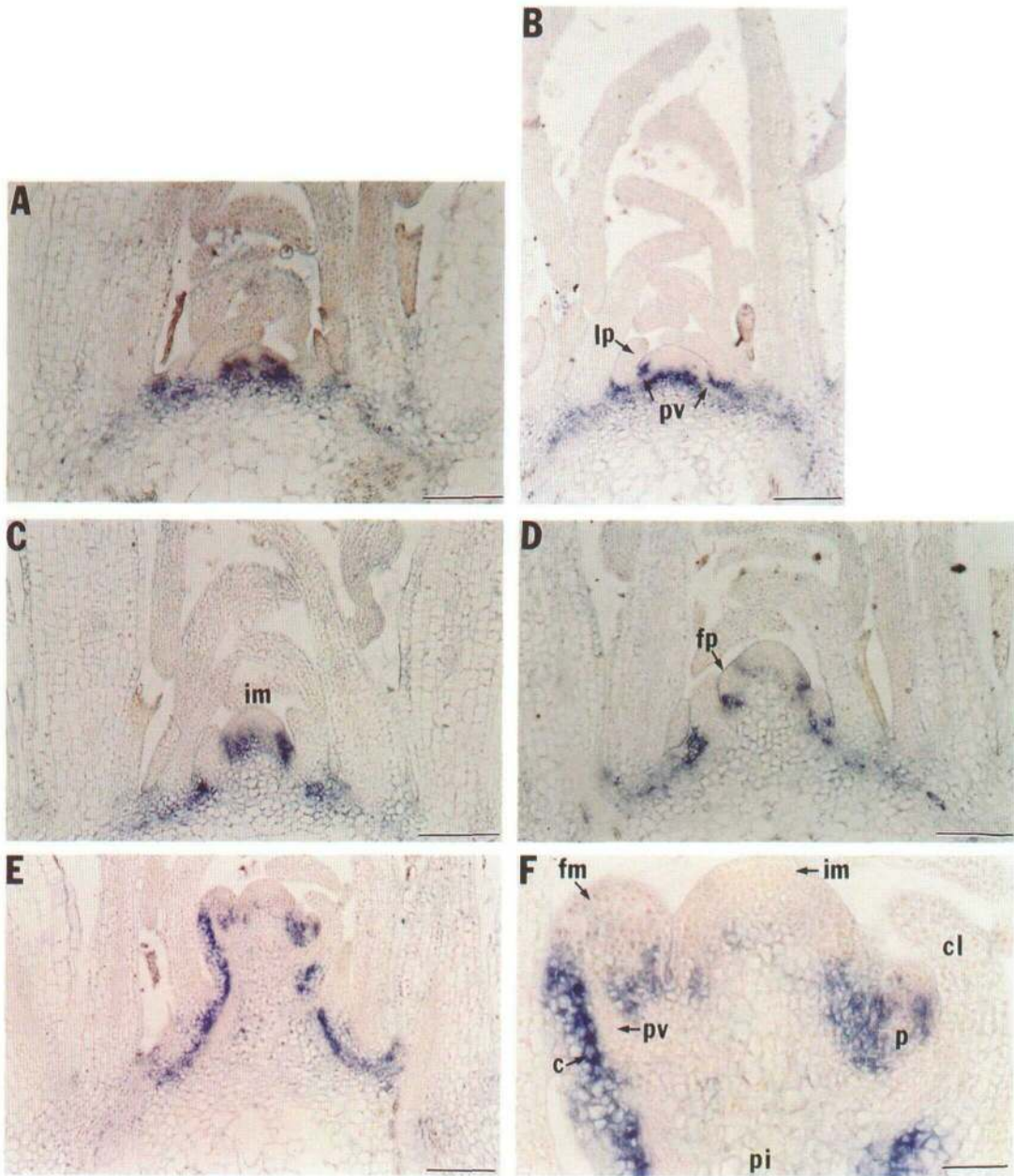
Longitudinal sections were hybridized to digoxigenin-labeled antisense probes.

(A) and (B) Serial sections (8  $\mu$ m thick) through 6-day-old seedlings grown in 16 hr of light. Bar in (B) = 100  $\mu$ m.

(C) and (D) Serial sections (8  $\mu$ m thick) through the shoot apex of an  $\sim$ 23-day-old plant grown in 8 hr of light. Bar in (D) = 100  $\mu$ m.

(E) and (F) Higher magnifications of sections shown in (C) and (D), respectively. Bar in (F) = 30  $\mu$ m.

In (F), the arrow indicates a group of cells that are not expressing *KNAT1*. These cells may correlate with a site of leaf initiation. cz, central zone; h, hypocotyl; lp, leaf primordium; m, meristem; pz, peripheral zone; rz, rib zone; s, stipule; sr, subapical region; v, vascular tissue.



**Figure 7.** Distribution of *KNAT1* mRNA in the Shoot Apex during Transition-to-Flowering Stages.

All sections are longitudinal and median or close to median and were hybridized to digoxigenin-labeled antisense probes. Plant growth conditions and stages are as described by Hempel and Feldman (1994); hours indicate time points after an inductive 16-hr photoperiod (hour 0 is the time point midway [8 hr] through the first inductive time period).

(A) Hour 16 after floral induction.

(B) Hour 40 after floral induction.

(C) Hour 64 after floral induction.

(D) Hour 88 after floral induction.

(E) Hour 136 after floral induction.

(F) Higher magnification of the inflorescence and floral meristem shown in (E).

In (A) to (E), bars = 100  $\mu$ m. In (F), bar = 30  $\mu$ m.

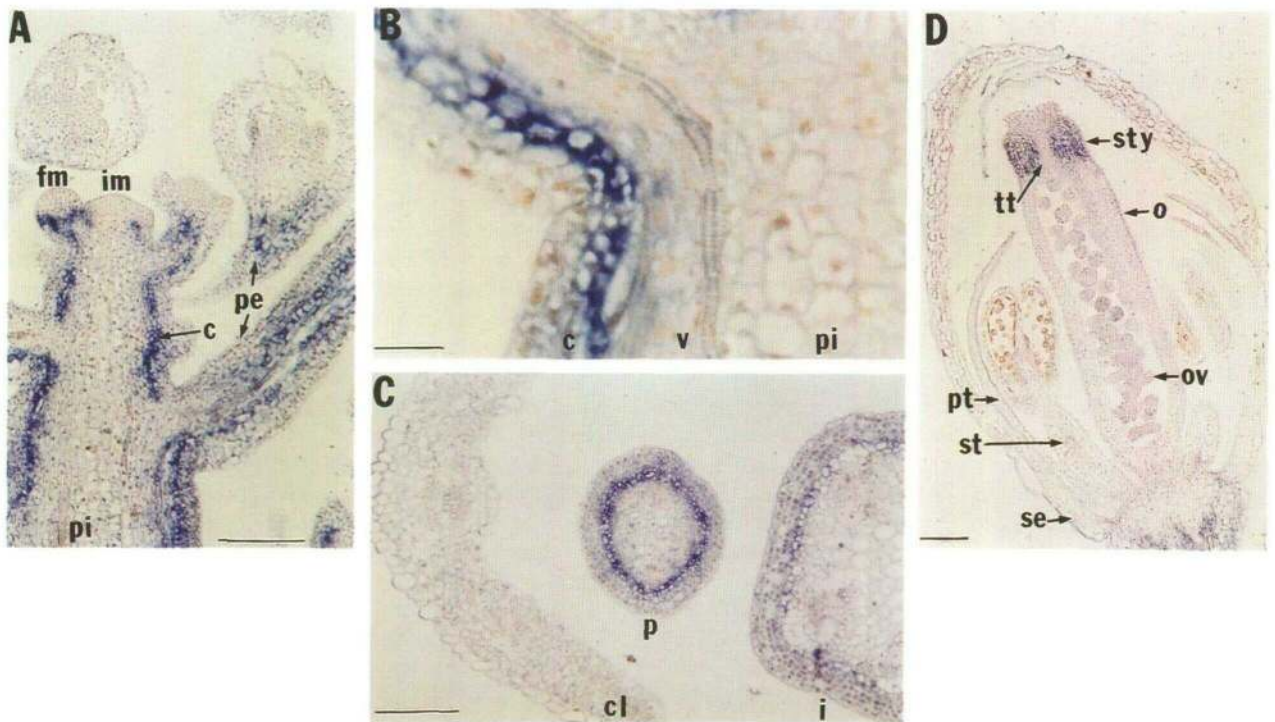
c, cortex; cl, cauline leaf; fp, floral primordium; im, inflorescence meristem; lp, leaf primordium; p, paraclade; pi, pith; pv, provascular trace.



of the apex, is shown in Figure 7B. At this stage, *KNAT1* expression became restricted to the lower portion of the apex; there was no detectable message in the inflorescence meristem. Expression continued at the base of developing leaves, but was excluded from provascular strands associated with the shoot apex. By hour 64, elongation of the inflorescence axis was beginning to occur (Figure 7C). By hour 88, floral primordia could be seen (Figure 7D). At these stages, *KNAT1* expression was confined mainly to the outer cell layers or cortex of the primary inflorescence stem. A low level of expression could be seen at the base of the inflorescence meristem and, depending on the plane of the section, in cells encircling the base of the floral primordia (Figure 7D). The *KNAT1* transcript was absent from the pith and from the base of leaf primordia associated with developing paraclades (Figures 7C to 7E). As elongation of the primary inflorescence axis proceeded, the *KNAT1* message became restricted to the cortical cell layers adjacent to the developing vascular tissue of the stem (Figures 7E and 7F). As shown in Figure 7F, expression extends into the cortex of the developing pedicel and paraclade, but stops abruptly at the base of the flower and lateral inflorescence meristems.

The pattern of *KNAT1* expression in the inflorescence remained constant throughout flowering (compare Figures 7E and 8A). The *KNAT1* message was localized primarily to the inner layers of the cortex along the length of the primary inflorescence stem and in the elongating stems of paraclades (Figures 8A to 8C). The *KNAT1* message was not detected in cauline (bract) leaves (Figure 8C). The *KNAT1* transcript was absent from the outer three whorls (sepals, petals, and stamens) of floral organs at all stages of development (Figures 8A and 8D) (for a description of stages, see Smyth et al., 1990). However, *KNAT1* mRNA was detected in the fourth floral whorl in the region that will become the style. Expression was first seen at stage 8 and continued until stage 12. In the style, a ring of expression occurred in cells surrounding the transmitting tissue (Figure 8D). For all in situ hybridization experiments performed on inflorescence and floral tissues, control sections hybridized with a sense probe showed no signal above background (data not shown).

To investigate the pattern of *KNAT1* expression during embryogenesis, in situ hybridization experiments were performed using longitudinal sections of embryos at the early-heart, late-



**Figure 8.** *KNAT1* Expression in Inflorescence and Flowers.

All sections were hybridized to digoxigenin-labeled antisense probes.

(A) Longitudinal section through primary inflorescence. Bar = 100  $\mu$ m.

(B) Higher magnification of inflorescence stem shown in (A). Bar = 20  $\mu$ m.

(C) Transverse section through primary inflorescence and paraclade. Bar = 100  $\mu$ m.

(D) Longitudinal section through stage 12 flower. Bar = 100  $\mu$ m.

c, cortex; cl, cauline leaf; fm, floral meristem; im, inflorescence meristem; i, primary inflorescence; o, ovary; ov, ovule; p, paraclade (secondary inflorescence); pe, pedicel; pi, pith; pt, petals; se, sepals; st, stamens; sty, style; tt, transmitting tissue; v, vascular tissue.

heart, linear-cotyledon, and curled-cotyledon stages (West and Harada, 1993). A hybridization signal above background was not detected in embryos at any of these stages, indicating that *KNAT1* mRNA is either not detectable owing to low abundance or not present during mid to late embryo development (data not shown).

### Altered *KNAT1* Expression Affects Arabidopsis Development

The *KNAT1* coding sequence was transcriptionally fused to the constitutive 35S promoter from cauliflower mosaic virus (CaMV), and the 35S-*KNAT1* expression construct was transformed into Arabidopsis. The CaMV 35S promoter is active in most cell types, although the level of activity is variable in different tissues (Benfey and Chua, 1990). Only five independent 35S-*KNAT1* transgenic lines were recovered and characterized owing to a low regeneration frequency and a reduction in the fertility of T<sub>1</sub> plants. The T<sub>2</sub> generation for all five lines was characterized with respect to differences in phenotype. Two of the five transformed lines exhibited extreme alterations in morphology; one line had less severe morphological defects, and the remaining two lines resembled wild-type plants in their growth and development. Segregation of kanamycin-resistant (Kan<sup>r</sup>) and kanamycin-sensitive (Kan<sup>s</sup>) plants in the T<sub>2</sub> generation was used to estimate the number of unlinked T-DNA insertions present in transgenic plants. A 3:1 segregation of Kan<sup>r</sup> seedlings in the progeny of the most extremely altered lines, E17 and B6, indicated that each line contains one or more transgenes at a single genomic locus. As a control, transformed plants carrying a 35S- $\beta$ -glucuronidase (*GUS*) construct were also generated. The 35S-*GUS* transformants were normal in phenotype (data not shown).

The most dramatic phenotypic changes observed in the *KNAT1* transformants were defects in leaf development. Vegetative growth in wild-type Arabidopsis is characterized by changes in leaf shape or heteroblasty as the plants pass from juvenile to adult stages. The first two leaves produced by the juvenile vegetative meristem are small, round, and entire, whereas later adult leaves are larger, spatulate, and serrate (Bowman, 1994). In the class of *KNAT1* transformants with the most extreme phenotype, the first two leaves were normal in shape. However, all subsequent rosette leaves were highly lobed, and their shape differed significantly from that of wild-type leaves, as shown in Figure 9. The pattern of lobing within a single leaf was irregular or asymmetrical across the midvein. There was a great deal of variability in the degree and pattern of lobing both within a single plant and among different transformants. In addition to lobing, the leaves were usually curled and/or wrinkled. Similar defects were seen in cauline (bract) leaves of transgenic plants (Figure 9C). In some transgenic plants, it was difficult to distinguish the petiole from the upper blade portion of rosette leaves (Figures 9A and 9D). The most severely affected *KNAT1* transformants also exhibited defects in floral development. Elongation of sepals, petals, and

stamens was abnormal or reduced compared with wild-type flowers (data not shown). Most aspects of carpel formation appeared normal; however, in some flowers, carpels exhibited a slightly bent or distorted appearance. Lines with the most severe floral defects produced fewer siliques. The reduction in fertility observed in the transgenic plants may have resulted from abnormal stamen and/or carpel development.

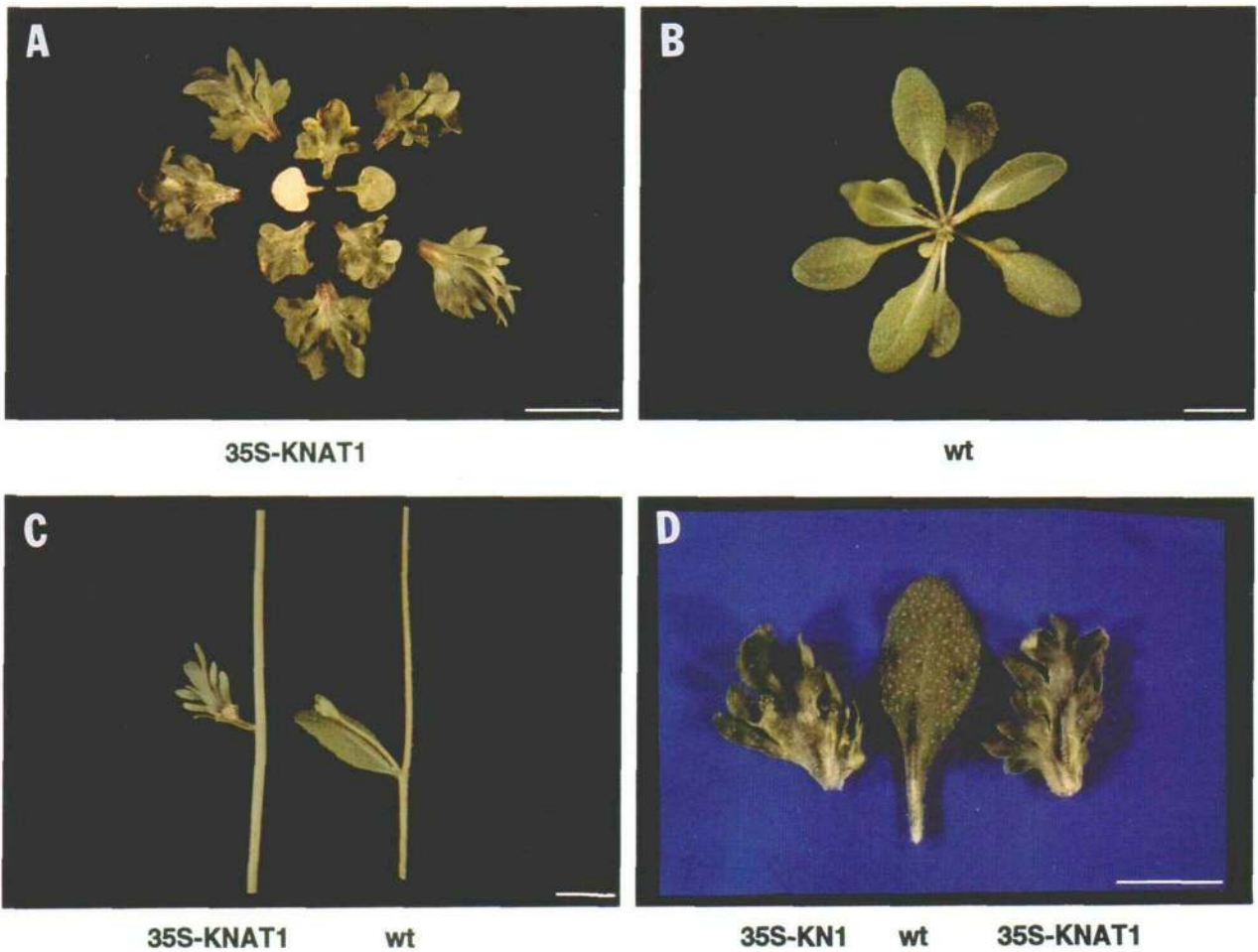
RNA gel blot analysis was used to examine the levels of *KNAT1* transcript in representative transgenic lines. In wild-type plants, *KNAT1* expression was not detected in leaves at various developmental stages (Figures 5A and 6A to 6F). Figure 10 shows a 1.6-kb transcript in mature rosette leaves of the two independent transgenic lines, E17 and B6, that exhibited abnormal leaf morphology, including leaf lobing. In contrast to the two *KNAT1* transcripts seen in flowers, inflorescences, and vegetative shoot apices of wild-type plants (Figure 5A), a single transcript of  $\sim$ 1.6 kb in leaves of transgenic plants was expected based on the length of the *KNAT1* cDNA insert used in preparing the 35S-*KNAT1* construct (see Methods). No hybridization was seen in RNA isolated from a transgenic line (line C38), which was normal with respect to leaf phenotype (Figure 10A, lane 3). The results of DNA gel blot analysis suggested that the 35S-*KNAT1* insertion in line C38 has undergone some type of rearrangement (data not shown).

### Expression of the Maize *kn1* Gene Product in Arabidopsis

We were interested in determining whether expression of the *kn1* gene product in Arabidopsis would result in phenotypes similar to those of 35S-*kn1* transgenic tobacco plants (Sinha et al., 1993) and how these phenotypes would compare with Arabidopsis plants transformed with the 35S-*KNAT1* construct. A 35S-*kn1* chimeric gene was introduced into Arabidopsis, and the resulting transgenic plants were examined for phenotypic alterations. A total of 11 independent T<sub>1</sub> lines were obtained. The T<sub>2</sub> progeny from all lines was examined for changes in plant morphology. Four T<sub>2</sub> lines had highly abnormal morphological phenotypes, whereas the remaining seven lines were less severely affected or normal in phenotype. The phenotype of the most severely affected class of *kn1* transformants resembled the phenotype of the 35S-*KNAT1* transformants in several ways: rosette and cauline leaves were curled, wrinkled, and/or mildly to deeply lobed (Figure 9D), and the size and shape of floral organs, particularly petals and stamens, were reduced or abnormal (data not shown).

To determine the relative levels of *kn1* expression in the 35S-*kn1* transformants, RNA was isolated from mature rosette leaves of T<sub>2</sub> plants with either severe or normal phenotypes. In leaves from three different T<sub>2</sub> lines that displayed mild to severe leaf phenotypes, a 1.6-kb transcript was detected on RNA gel blots hybridized with a *kn1* probe (Figure 10B, lanes 3, 4, and 5). No signal was detected in RNA extracted from a transformed line that had normal leaves (Figure 10B, lane 2) or from wild-type plants (Figure 10B, lane 1).





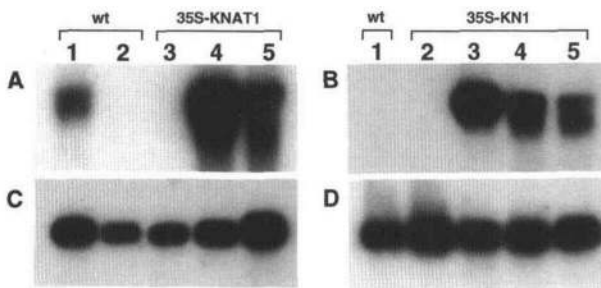
**Figure 9.** Phenotypes of Arabidopsis Plants Overexpressing *KNAT1* or the Maize *kn1* Gene.  
**(A)** Rosette from a 35S-*KNAT1* transformant (line B6). Leaves were detached to facilitate viewing of the lobed phenotype of each individual leaf. The two nonlobed leaves are the first leaves initiated.  
**(B)** Rosette from a wild-type (wt) plant.  
**(C)** Comparison of cauline leaves from a 35S-*KNAT1* transformant (line B6) and the wild type (wt).  
**(D)** Comparison of rosette leaf phenotypes from a 35S-*kn1* transformant (line J2-25), the wild type (wt), and a 35S-*KNAT1* transformant (line B6). Bars = 1 cm.

**DISCUSSION**

***KNAT1* and *KNAT2* Are Members of a Large Family of *kn1*-like Homeobox Genes**

In this report, we describe the characterization of two genes, *KNAT1* and *KNAT2*, encoding KN1-like homeodomain proteins in Arabidopsis. *KNAT1* and *KNAT2* are members of a large family of *kn1*-like genes that have been identified in several plant species including maize (Vollbrecht et al., 1991), rice (Matsuoka et al., 1993), tomato (N. Sinha and S. Hake, unpublished data), sunflower (D. Jackson and S. Hake, unpublished data), and

soybean (Ma et al., 1994). Within the homeodomain, the *KNAT1* and *KNAT2* proteins share several characteristic features with other members of the KN1-like family. Both predicted proteins have an invariant core sequence of 13 amino acids in the third or recognition helix, a conserved intron position in the second helix, and a three-amino acid insertion between the first and second helices of the homeodomain. Immediately adjacent to the homeodomain of *KNAT1* and *KNAT2*, a series of 24 amino acids that contains repeating hydrophobic residues (the ELK domain) is also conserved. This novel motif is present in all KN1-like proteins that have been identified. The conserved features present in the extended homeodomain regions of *KNAT1*, *KNAT2*, and other KN1-like proteins distinguish their



**Figure 10.** Expression of *KNAT1* and *kn1* Transcripts in Arabidopsis Transformed with 35S-*KNAT1* or 35S-*kn1* Constructs.

(A) RNA gel blot analysis of *KNAT1* transgenic lines. Total RNA was isolated from inflorescence tissue (lane 1) or rosette leaves (lanes 2 to 5) from the wild type (wt; lanes 1 and 2) and the independent 35S-*KNAT1* transgenic line C38 (lane 3), line E17 (lane 4), and line B6 (lane 5). Eight micrograms of RNA was loaded per lane. The blot was probed with a 5' portion of the *KNAT1* cDNA.

(B) RNA gel blot analysis of *kn1* transgenic lines. Total RNA was isolated from rosette leaves of the wild type (wt; lane 1) and the independent 35S-*kn1* transgenic line J15 (lane 2), line J3-2 (lane 3), line J1-29 (lane 4), and line J2-25 (lane 5). Ten micrograms of RNA was loaded per lane. The blot was probed with the *kn1* homeobox.

(C) Control hybridization. The blot in (A) was reprobed with *ROC1* (Lippuner et al., 1994) to show relative RNA loading.

(D) Control hybridization. The blot in (B) was reprobed with *ROC1* to show relative loading.

respective genes from other types of homeobox genes reported in plants (Bellmann and Werr, 1992; Mattsson et al., 1992; Schena and Davis, 1992; Carabelli et al., 1993; Schena et al., 1993; Schindler et al., 1993; Korfhage et al., 1994; Rerie et al., 1994).

The *kn1*-like gene family in maize (*knox* genes) consists of approximately 12 genes that have been subdivided into two classes based on sequence similarity within the homeodomain (Kerstetter et al., 1994). Class 1 *knox* genes are more similar to *kn1* (73 to 89% identity in the homeodomain) compared with class 2 genes (55 to 58% identity with the KN1 homeodomain). Based on genetic evidence, two class 1 *knox* genes correspond to the dominant morphological mutations *Rough sheath1* (*Rs1*) and *Liguleless3* (*Lg3*) (P. Becraft, J. Fowler, and M. Freeling, personal communication). Both mutants are phenotypically similar to *Kn1* (Freeling, 1992). The extended homeodomain regions of *KNAT1* and *KNAT2* share a high degree of identity with class 1 *knox* genes. Within this class, the *KNAT1* homeodomain is more similar to the homeodomain encoded by the *knox* gene *rs1* (95% identity) than it is to the KN1 homeodomain (89% identity). An alignment of the complete amino acid sequences of *KNAT1* and *RS1* reveals that these two predicted proteins share an overall identity of ~61%, which is slightly higher than the sequence identity between *KNAT1* and KN1 (~53%). The intron-exon structure of *KNAT1* is almost identical

to both that of *kn1* and *rs1* (P. Becraft, R. Schneeberger, S. Hake, and M. Freeling, manuscript submitted). Based on shared amino acid substitutions within the homeodomain, *KNAT2* appears to be more closely related to the class 1 genes *knox5*, *knox11*, and *lg3*. Outside the extended homeodomain-encoding region of each *KNAT* gene, sequence homology is less well conserved. The 5' coding regions of *KNAT1* and *KNAT2* differ significantly from those of *kn1*, *rs1*, and several other class 1 *knox* genes. Three *kn1*-like Arabidopsis genes (*KNAT3*, *KNAT4*, and *KNAT5*), which are more similar to class 2 *knox* genes, have also been identified (K. Serikawa and P. Zambryski, unpublished data).

### ***KNAT1* and the Maize Class 1 *knox* Genes Have Similar Vegetative Expression Patterns**

*KNAT1* and *KNAT2* are expressed in several different tissues during vegetative and reproductive phases of growth. During vegetative development, the expression pattern of *KNAT1* in Arabidopsis closely resembles the expression patterns of several class 1 *knox* genes in maize. Based on RNA gel blot analysis, the class 1 genes *kn1*, *rs1*, *knox3*, *knox4*, and *knox8* are all strongly expressed in shoot meristem-enriched tissues, but are not detected in leaves (Kerstetter et al., 1994). In situ hybridization has shown that *kn1*, *rs1*, *knox3*, and *knox8* are expressed in the unexpanded stem and exhibit distinct, but overlapping domains of expression in the shoot apical meristem (Jackson et al., 1994). In the vegetative shoot apex of Arabidopsis, *KNAT1* expression was seen in the meristem and in a region just proximal to the meristem that extends to the base of several newly initiated leaf primordia. In shoot meristems of plants grown under short-day conditions, the *KNAT1* message was reduced or absent in discrete groups of cells located on the flanks of the meristem. No expression was detected in leaf primordia, even at very early stages of primordium formation. *KNAT1* transcript was also present in the unexpanded stem of the rosette, where it appeared in cells flanking but not within provascular traces. Within the vegetative meristem, *KNAT1* message was more abundant in the peripheral and rib zones than in the central zone. In this respect, *KNAT1* is more similar to *rs1* and *knox3*, which both show more restricted patterns of expression in the maize shoot meristem (Jackson et al., 1994).

Although it is difficult to make direct comparisons between the expression patterns of *KNAT1* in Arabidopsis and those of class 1 *knox* genes in maize, there are two significant features of the expression patterns that appear to be conserved. The Arabidopsis and class 1 maize genes are all expressed in the vegetative meristem, and each gene is selectively down-regulated at the same time as or immediately prior to leaf primordium initiation. Predicting the location of incipient leaf primordia is easier in longitudinal sections of the maize shoot meristem than in Arabidopsis because the phyllotaxy is distichous and the approximate number of leaf founder cells has



been determined (Poethig, 1984). The down-regulation of *kn1* clearly accompanies leaf initiation sites in maize (Jackson et al., 1994). In Arabidopsis, the first two leaves are initiated opposite to one another, whereas all subsequent leaves as well as flowers are initiated in a spiral phyllotaxy (Bowman, 1994). Similar to other dicots, Arabidopsis leaves develop from three to four cell layers on the flanks of the shoot apical meristem; however, the number of meristematic cells recruited into the leaf primordium has not been determined. It is possible that the region of the shoot meristem lacking *KNAT1* expression (Figures 6D and 6F) corresponds to the site of incipient leaf primordium formation similar to what we find for *kn1* in the maize meristem.

We have proposed that in wild-type plants, *kn1* functions to maintain the indeterminate state of plant meristems (Smith et al., 1992; Sinha et al., 1993). This hypothesis is based on the meristem expression patterns just mentioned and observations of ectopic expression of *kn1* in leaves. Such ectopic *kn1* expression, conditioned either by gain-of-function mutations in maize or by transgenic overexpression experiments, leads to the adoption of new cell fates that range from other leaf cell identities to the production of ectopic meristems. The unique aspects of the expression domains of *rs1*, *knox3*, and *knox8* in the maize shoot meristem suggest that these genes also play potentially distinct roles in the meristem. Again, dominant mutations in at least two class 1 *knox* genes, *rs1* and *lg3*, result in leaf phenotypes similar to that conferred by *kn1*, which also involve cell fate transformations in leaves (Freeling, 1992).

Because homeobox gene function is often conserved between organisms as different as mice and *Drosophila* (McGinnis and Krumlauf, 1992), it is tempting to speculate that class 1 *kn1*-like genes in Arabidopsis also play a role in meristem function. The expression pattern of *KNAT1* in the vegetative shoot meristem fits such expectations. Significantly, a class 1 *KNAT* gene that is 84% identical to *KNAT1* within the extended homeodomain region has been shown to correspond to the mutation *shoot meristemless1* (*stm1*) in Arabidopsis (K. Barton, personal communication). Characterization of the recessive phenotype conferred by *stm1* suggests that the wild-type gene product is required for initiation of the shoot meristem during embryonic development (Barton and Poethig, 1993). Determining when and where *STM1* is expressed during various stages of wild-type plant development will be important. The earliest time of *KNAT1* expression in the shoot meristem has not been accurately determined. However, in the mature embryo, *KNAT1* transcript either was not present or was present at levels too low to be detected by the in situ hybridization techniques used here. Based on RNA gel blot analysis, the expression pattern of *KNAT2* in both vegetative and floral tissues was very similar to that of *KNAT1*. Experiments are now underway to examine the localization of *KNAT2* mRNA in various tissues. *STM1*, *KNAT1*, and *KNAT2* may all be expressed in the shoot meristem, but in different domains or at different times, which would suggest that each gene has a unique function during vegetative development.

### Loss of *KNAT1* Meristem Expression Coincides with Floral Induction.

In contrast to the similar vegetative expression patterns seen with *KNAT1* and the class 1 *knox* genes, the distribution of *KNAT1* transcript in the inflorescence differs significantly. In the inflorescence, *KNAT1* expression is limited to specific cells within the cortex of the stem. This ring of *KNAT1* expression is located immediately adjacent to the vascular cylinder. *KNAT1* is absent from all floral organ primordia, but is present in the style. The function of *KNAT1* in these apparently distinct cell types is not clear. *kn1*, *rs1*, *knox3*, and *knox8* are expressed at high levels throughout the corpus of the inflorescence and floral meristem. These genes are not expressed in any lateral organs and disappear before the organs form (Jackson et al., 1994). Thus, *KNAT1* is similar in its exclusion from cauline leaves, petals, sepals, and anthers but differs from the maize genes in its absence from both inflorescence and floral meristems. The similarity in the expression patterns of *KNAT1* and several class 1 *knox* genes in the vegetative shoot meristem suggests that these genes play similar roles during vegetative development and that this function has been conserved. However, the function(s) of *KNAT1* and the maize *knox* genes may be quite distinct during floral development.

Expression of *KNAT1* in the meristem disappears at the same time the shoot apex undergoes the transition from vegetative to reproductive development. In angiosperms, this transition is a critical developmental event. Consequently, floral induction is regulated by a number of different developmental and environmental signals, including plant age, photoperiod, and temperature (reviewed in Bernier et al., 1994). The vegetative-to-inflorescence transition is characterized by changes in meristem structure, cellular metabolism, and gene expression (Bernier et al., 1981; Meeks-Wagner, 1994). Genes that display a modified pattern of expression during this transition may play an important role in specifying the developmental programs unique to either vegetative or inflorescence meristems. Arabidopsis mutants, such as *embryonic flower* (*emf*), that are disrupted in the transition from vegetative to reproductive development have been identified (Sung et al., 1992). *emf* mutant embryos flower precociously, producing inflorescence shoots directly upon germination. Based on the mutant phenotype conferred by *emf*, Sung et al. (1992) proposed that reproductive development is the basal or default state of the shoot apex in Arabidopsis and that negative regulation of the reproductive program is necessary for normal vegetative development to occur. Perhaps *KNAT1* functions with *EMF*, or is regulated by *EMF*, to maintain the vegetative state of the shoot meristem.

In the early floral meristem (flower primordia at stages 1 and 2), the expression pattern of *KNAT1* is the reciprocal of the pattern exhibited by the floral meristem identity gene *LEAFY* (*LFY*) and the floral organ identity genes *APETALA1* (*AP1*) and *AP2* (Mandel et al., 1992; Weigel and Meyerowitz, 1993; Jofuku et al., 1994). *KNAT1* expression in flower primordia at stage 2 occurs in the developing pedicel; the region of expression extends

up to and includes cells immediately below the rib meristem. In young flower primordia, *LFY*, *AP1*, and *AP2* transcripts are present throughout the meristem, but expression stops at the base of the primordia where *KNAT1* expression begins. It is possible that regulatory interactions between *KNAT1* and *LFY*, *AP1*, and/or *AP2* dictate these expression patterns.

### Ectopic *KNAT1* Expression Alters Leaf Development in Arabidopsis

To gain further insight into the possible function of *KNAT1*, we transformed Arabidopsis plants with a construct containing the *KNAT1* cDNA under the control of the CaMV 35S promoter. Disruption of the concentration, timing, and/or tissue specificity of *KNAT1* expression altered the morphology of both leaves and flowers. In the most severe cases, ectopic expression of *KNAT1* in leaves dramatically altered leaf shape, resulting in highly lobed leaves instead of the characteristic entire leaf shape of wild-type plants. A similar leaf phenotype was observed in Arabidopsis plants overexpressing the maize *kn1* gene. Similarities can be drawn between the transformants and the mutant phenotypes conferred by *Kn1* and *Rst1* in maize, which result from ectopic expression in leaves. Leaf blade cells of these maize mutants adopt cell fates normally restricted to the sheath or basal portion of the leaf (Freeling, 1992; Becraft and Freeling, 1994; Sinha and Hake, 1994). In at least one 35S-*KNAT1* transformant, lobed leaves contained ectopic stipules, which are also normally positioned at the basal region of the leaf (G. Chuck and S. Hake, unpublished data).

Tobacco plants transformed with a 35S-*KNAT1* construct display ectopic formation of shoots on leaf surfaces in addition to leaf lobing (Sinha et al., 1993). Ectopic shoot formation has not been observed in Arabidopsis plants constitutively expressing *KNAT1* or *kn1*. However, tobacco plants transformed with the 35S-*KNAT1* construct have severe alterations in leaf morphology, including leaf lobing and ectopic shoot formation (J. Long, unpublished data). The parallel phenotypes produced by overexpressing *KNAT1* and *kn1* in Arabidopsis and tobacco suggest that these two genes are capable of regulating similar sets of downstream genes. It will be interesting to see whether other class 1 maize genes induce morphological transformations similar to those induced by *kn1* when overexpressed in Arabidopsis or tobacco. Formation of ectopic shoots on leaf blades in tobacco *KNAT1* and *kn1* transformants supports the hypothesis that class 1 genes are involved in maintaining cells in an undifferentiated, meristematic state. In the transformants, continuous expression of *KNAT1* or *kn1* may disrupt the normal differentiation processes occurring in developing leaves such that groups of leaf cells acquire characteristics of apical meristems.

To understand how misexpression of *KNAT1* in the 35S-*KNAT1* transformants results in abnormal leaf morphology, it will be important to determine when during leaf development differences in leaf shape become apparent. The Arabidopsis transformant phenotypes suggest that changes in leaf shape

occur later, after leaf primordia have formed. Ectopic expression of *KNAT1* in developing leaves may alter growth patterns or the timing of cell differentiation within the leaf blade. In wild-type plants, young developing leaves are composed largely of rapidly dividing, undifferentiated cells. Differentiation of mesophyll cells and cessation of mitotic activity begin at the leaf tip and proceed toward the base. Older leaves retain a zone of mitotic cells in the lower portion of the leaf (Pyke et al., 1991). The presence of the *KNAT1* gene product in developing leaves where the gene is not normally expressed may directly or indirectly alter the pattern of differentiation or interfere with cell division control. Localized areas of restricted or unchecked growth could produce lobing and puckering of leaf blades.

Based on sequence comparison and expression patterns, *KNAT1* and *KNAT2* belong to a subfamily of *kn1*-like homeobox genes (designated class 1) that appear to play a role in shoot meristem organization. It is difficult to identify specific maize homologs for *KNAT1* and *KNAT2* on the basis of sequence similarities because they are similar to a number of maize class 1 genes. In addition, the vegetative expression pattern of *KNAT1* resembles that of more than one class 1 *knox* gene. Ectopic expression of *KNAT1* disrupts normal leaf development and may cause alterations in the patterns of leaf cell differentiation consistent with models for class 1 *knox* gene action in maize. Although *KNAT1* and *kn1* produce similar phenotypes when overexpressed in transgenic plants, this result does not necessarily imply that these two genes are functionally homologous. Identification of loss-of-function phenotypes corresponding to each of the maize class 1 genes should help elucidate the role of individual genes during development. In Arabidopsis, an antisense approach is being used to generate loss-of-function phenotypes for *KNAT1* and *KNAT2* and, when compared with similar phenotypes in maize, may help resolve the question of class 1 gene homology in these two plant species. Many important questions regarding the function of *KNAT1* and related genes in Arabidopsis remain to be answered. For example, how is *KNAT1* expression regulated in the shoot meristem and what downstream genes are targets for *KNAT1*? The striking leaf phenotypes observed in 35S-*KNAT1* transformants can now be used to isolate suppressor mutations that may identify interacting genes.

## METHODS

### Plant Growth Conditions

*Arabidopsis thaliana* (Columbia ecotype unless stated otherwise) seedlings were grown in vitro on germination medium (Murashige and Skoog salts and vitamins supplemented with 10 g/L sucrose and 0.8% agar; Murashige and Skoog, 1962; Valvekens et al., 1988) under a 16-hr photoperiod (16-hr light/8-hr dark) or in total darkness at 22°C. Using green safelights, dark-grown seedlings were harvested and frozen in liquid nitrogen. Roots were harvested from 3- to 4-week-old plants grown in liquid germination medium that was constantly agitated. For collection

of leaves, stems, flowers, and siliques, plants were grown in the greenhouse under continuous illumination at 21°C day/18°C night. To delay initiation of floral primordia, plants were grown in 8-hr photoperiods (8-hr light/16-hr dark) at 22°C. After 23 to 27 days of growth in 8-hr photoperiods, flowering was induced by successive 16-hr photoperiods. These conditions result in the rapid and synchronous initiation of flower formation in Arabidopsis populations (Hempel and Feldman, 1994).

### Screening of cDNA and Genomic Libraries

*KNAT1* and *KNAT2* (*knotted*-like from *Arabidopsis thaliana*) cDNA clones were initially obtained by screening a  $\lambda$ ZAPII (Stratagene) cDNA library constructed with poly(A)<sup>+</sup> RNA from 2-week-old plants of the Wassilewskija ecotype. The probe used to screen the cDNA library was a fragment of the maize *knotted1* (*kn1*) gene extending from the sequence encoding the ELK region to the end of the homeodomain (Vollbrecht et al., 1991, 1993). Low-stringency conditions for plaque hybridization (9 × SSC [1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate], 57°C) and washes (1 × SSC, 57°C) were used. Longer cDNA clones were isolated by screening a  $\lambda$ ZAPII library prepared from seedling (ecotype Columbia) poly(A)<sup>+</sup> RNA and a  $\lambda$ gt10 library prepared from RNA isolated from floral tissues (ecotype Landsberg). The seedling cDNA library was a gift from J. Ecker (University of Pennsylvania, Philadelphia, PA); the floral library was a gift from J. Bowman and E. Meyerowitz (California Institute of Technology, Pasadena, CA). The probes used to screen the cDNA library were 5' fragments of the previously identified *KNAT1* and *KNAT2* cDNA clones. Probes were labeled with <sup>32</sup>P-dCTP using a Multiprime Labeling kit (Amersham). High-stringency hybridization and washes were performed at 68°C. Phage isolates were converted to plasmid clones following the Stratagene  $\lambda$ ZAP excision protocol. Genomic clones corresponding to *KNAT1* and *KNAT2* were isolated from an Arabidopsis (ecotype Columbia) genomic library in  $\lambda$ GEM11 (Promega). The genomic library was a gift from R. Davis (Stanford University, Stanford, CA). DNA inserts from purified phage isolates were subcloned into pGEM-7Z(+) (Promega).

### DNA Sequence Analysis

Dideoxy sequencing of cDNA and genomic clones was performed using a Sequenase kit, Version 2.0 (United States Biochemical), and specific oligonucleotide primers. Degenerate primers derived from the *kn1* homeobox sequence have been described by Kerstetter et al. (1994). Double-stranded DNA was used as a template and sequenced in both directions with the exception of intron sequences, which were sequenced only in one direction. Analysis of DNA and amino acid sequences was performed using the IG Molecular Biology Software System Release 5.4 (IntelliGenetics, Inc., Mountain View, CA) and National Center for Biotechnology Information BLAST E-MAIL server.

### RNAse Protection Analysis

The 5' ends of the *KNAT1* and *KNAT2* transcripts were determined using the Ambion (Austin, TX) RPAII RNase protection kit according to the manufacturer's instructions. For *KNAT1*, 450-bp and 578-bp genomic fragments were subcloned and in vitro transcribed. The resulting probe was hybridized to total RNA and digested with RNase, and the size of the protected product was determined on a 6% sequencing gel. For *KNAT2*, the same procedure was followed except a 244-bp genomic fragment was used.

### DNA Gel Blot Analysis

DNA was isolated from leaf or seedling tissue using the procedure of Dellaporta et al. (1983). Restriction digests of ~3  $\mu$ g of DNA were performed, and the resulting fragments were separated in a 1% agarose gel and blotted onto a Magna nylon transfer membrane (Micon Separations, Inc., Westboro, MA). High-stringency hybridizations were performed at 68°C in a solution containing 7% SDS and 0.25 M NaH<sub>2</sub>PO<sub>4</sub> (Robbins Scientific Co., Sunnyvale, CA). Filters were washed in 0.2 × SSC, 0.2% SDS at 68°C. Low-stringency hybridizations were performed at 55°C in a hybridization buffer containing 1% BSA, 2 mM EDTA, 0.5 M NaHPO<sub>4</sub>, pH 7.2, and 7% SDS (Church and Gilbert, 1984). Filters were washed in 1 mM EDTA, 0.1 M NaHPO<sub>4</sub>, 5% SDS at room temperature and exposed to film. Additional washes at higher temperatures were performed if necessary.

### RNA Gel Blot Analysis

Total RNA from different tissues was isolated using a modified phenol-SDS method (Timpte et al., 1994). Poly(A)<sup>+</sup> RNA was isolated using a Poly-A-Tract mRNA isolation kit (Promega). RNA was glyoxylated, size fractionated in a 1% agarose gel, and transferred to a Magna nylon transfer membrane as previously described (Sambrook et al., 1989). Hybridization was performed at 42°C in a solution containing 50% formamide, 5 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4), 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), 2% SDS, and 1 mg/mL denatured salmon sperm DNA. Filters were first washed in 1 × SSPE, 0.5% SDS at room temperature followed by two washes in 0.2 × SSPE, 0.5% SDS at 68°C. The random primer-labeled *KNAT1* probe contained the 5' noncoding sequence and ~150 bp of the first exon. The 5' *KNAT2* probe contained sequence from exons 1, 2, and 3. The cytosolic cyclophilin gene *ROC1* was used as a loading control (Lippuner et al., 1994).

### In Situ Hybridization

Antisense and sense transcripts for *KNAT1* clones were synthesized and labeled in vitro with digoxigenin-11-UTP (Boehringer Mannheim) using T7 or T3 polymerase. In vitro transcription reactions were performed following the manufacturer's protocol, except the ratio of labeled to unlabeled UTP was 1:1. The cDNA clones used were a 1.4-kb cDNA containing the homeobox but lacking the 3' portion of *KNAT1* and a subclone containing a 700-bp XbaI fragment from the 5' portion of *KNAT1* lacking the homeobox. Both probes gave identical results. Riboprobe purification, hydrolysis, and quantification were performed as described in the Boehringer Mannheim Genius System user's guide.

Tissue samples were fixed in 4% formaldehyde overnight at 4°C (Jackson, 1991). The fixed tissue was passed through an ethanol series and Histoclear (National Diagnostics, Atlanta, GA) before being embedded in ParaplastPlus (Fisher Scientific, Pittsburgh, PA). Tissue sections (8  $\mu$ m thick) were mounted on ProbeOnPlus slides (Fisher Scientific). Section pretreatment and hybridization were performed according to Jackson (1991) with the following modifications: (1) immediately prior to protease treatment, slides were placed in 0.2 M HCl for 20 min, rinsed in H<sub>2</sub>O, and then incubated in 2 × SSC for 20 min followed by another H<sub>2</sub>O rinse; (2) for the protease treatment, 1 mg/mL proteinase K (Sigma) was used and slides were incubated for 30 min at 37°C; (3) riboprobes were used at a concentration of ~0.5 ng/ $\mu$ L per kb probe complexity. After hybridization, slides were washed

twice at high stringency ( $0.2 \times$  SSC,  $55^\circ\text{C}$ ) followed by two rinses in NTE (0.5 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA) at  $37^\circ\text{C}$ . The slides were incubated at  $37^\circ\text{C}$  in NTE containing 20 mg/mL RNase A for 30 min followed by several rinses in NTE and a final wash in  $0.2 \times$  SSC at  $55^\circ\text{C}$ . Blocking steps and detection of hybridized transcripts using anti-digoxigenin antisera conjugated to alkaline phosphatase (Boehringer Mannheim) were performed as described by Coen et al. (1990). Sections were passed through an ethanol series and Histoclear before mounting in Merckoglas (EM Science, Cherry Hill, NJ).

### Arabidopsis Transformation

Construction of 35S-*kn1*, which contains the maize *kn1* cDNA inserted between the cauliflower mosaic virus (CaMV) 35S promoter and the terminator region of the nopaline synthase gene (*NOS*), has been previously described (Sinha et al., 1993). A 35S-*KNAT1* expression construct was prepared by replacing the *kn1* sequence in the 35S-*kn1* plasmid with the coding region of *KNAT1*. Because a full-length cDNA clone was not available when the 35S-*KNAT1* construct was made, 5' and 3' fragments from two different cDNA clones were ligated to create the  $\sim 1.6$ -kb *KNAT1* insert used in the 35S-*KNAT1* plasmid. A construct consisting of the 35S promoter, the  $\beta$ -glucuronidase (*GUS*) gene, and the *NOS* terminator (Sinha et al., 1993) was used as a control in plant transformations. All constructs were cloned into the binary vector pBIN19 (Bevan, 1984). The expression plasmids 35S-*kn1*, 35S-*KNAT1*, and 35S-*GUS* were introduced into *Agrobacterium tumefaciens* LBA4404 using a modified freeze-thaw method (Hofgen and Willmitzer, 1988). Arabidopsis lines (ecotype Nossen) were transformed using the *Agrobacterium*-mediated root transformation method of Valvekens et al. (1988). Transformants were selected on medium containing 50 mg/L kanamycin. Primary transformants ( $T_1$ ) were allowed to self-cross, and the resulting  $T_2$  progeny were scored for segregation of kanamycin resistance. Kanamycin-resistant  $T_2$  seedlings were transferred to soil, and mature plants were examined for alterations in morphology. The presence of transgenes in  $T_2$  lines was confirmed using the polymerase chain reaction and/or DNA gel blot analysis.

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