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A dominant mutation in the maize homeobox gene, *Knotted-1*, causes its ectopic expression in leaf cells with altered fates

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Summary

Dominant mutations of the *Knotted-1* (*Kn1*) homeobox gene of maize alter the differentiation and growth of cells associated with leaf veins. By analyzing *Kn1* transcripts and KN1 protein, we show that the gene is not expressed at high levels during the development of wild-type leaves. Instead, *Kn1* is expressed in apical meristems of vegetative and floral shoots, and is downregulated as leaves and floral organs are initiated. *Kn1* is also expressed in relatively undifferentiated cells within developing vascular bundles, as well as ground tissue, in immature, unelongated axes of wild-type vegetative and floral shoots. In *Kn1-N2* mutant plants, quantitative, but not qualitative differences are apparent in *Kn1* transcripts and KN1 protein, consistent with previous observations that dominant *Kn1* mutations map to non-coding regions of the gene. *Kn1* is expressed ectopically in vascular bundles within developing mutant leaves in a pattern that correlates with the phenotypic alterations produced by the *Kn1-N2* mutation. Thus, *Kn1* apparently alters the fates of leaf cells in which it is ectopically expressed from an early stage of leaf development. Based on these observations, we hypothesize that *Kn1* functions in its wild-type context as a regulator of cell determination.

Key words: maize, shoot development, meristems, homeobox genes, *Knotted-1*.

Introduction

The elaboration of a plant body results from the activity of meristems, groups of dividing cells that generate all the organs and tissues of the plant. The shoot apical meristem is an organized unit of undifferentiated, slowly-dividing cells that maintains itself as it progressively initiates leaves and floral organs. The rapid, polar growth of these lateral organs is accompanied initially by a high rate of cell division compared to that within the apical meristem, but their potential for growth is limited, and they begin to differentiate shortly after initiation (reviewed in Steeves and Sussex, 1989). The developmental regulation of the shoot apical meristem, and the initiation and subsequent differentiation of lateral organs, are poorly understood. However, mutations altering plant development offer a unique opportunity to study the underlying mechanisms.

Dominant mutations of the *Knotted-1* (*Kn1*) gene of maize alter patterns of development in the leaf. *Kn1* was cloned by transposon tagging (Hake et al., 1989), and shown to encode a homeodomain protein (Vollbrecht et al., 1991). *Kn1* is thus the first higher plant gene to join a large family of homeobox genes in the animal kingdom that are associated with the control of development. Many (though not all) animal homeobox genes are believed to play a critical role in diverse processes in animal development, including the control of pattern formation in insect and vertebrate embryos, and the specification of cell fates in many tissues (for review, Gehring, 1987; Ingham, 1988; McGinnis and Krumlauf, 1992). The homeodomain itself is a DNA-binding domain; several homeodomain proteins have been shown to regulate transcription (for review, Hayashi and Scott, 1990).

Molecular analyses of several of the dominant *Kn1* mutations has shown that they are caused by changes in non-coding portions of the gene. The *Kn1-O* allele is caused by a 17 kb tandem duplication (Veit et al., 1990), in which the entire coding region is repeated, placing one transcription start site within 500 bp of the junction between repeats (Lowe et al., 1992). All other alleles so far analyzed are caused by or associated with transposon insertions into intron sequences (Hake et al., 1989; R. Walko, N. Sinha, B.G. and S.H., unpublished observations; Hake, 1992). Changes in non-coding regions of the gene are consistent with the dominant, "neomorphic" nature of *Kn1* mutations (Freeling and Hake, 1985), and suggest that the mutant phenotype results from altered regulation of the *Kn1* gene.

To gain an understanding of the role of the *Kn1* gene product in normal maize development and the cause of the mutant phenotype, we examined the expression of *Kn1* at the RNA and protein levels in wild-type and mutant plants.

Materials and Methods

Plant material

Wild-type maize plants were of the B73 inbred genotype (Pioneer Hi-Bred, Johnston, Iowa). Homozygous mutant plants and their normal siblings were selected from a family segregating the *Kn1-N2* mutation, obtained originally from G. Neuffer, University of Missouri, Columbia.

RNA preparation and northern blotting

Approximately 250 mg of tissue pooled from 6-8 individuals was ground under liquid nitrogen, mixed with 300 μl denaturing solution (5 M guanidine thiocyanate, 25 mM sodium citrate, 1% sarcosyl, 1% β-mercaptoethanol, 3 mg/ml diethyldithiocarbamate), extracted with phenol:chloroform (5:1), and precipitated with isopropanol. The resuspended pellet was extracted again with phenol:chloroform (1:1), and RNA was ethanol precipitated. Ten μg of total RNA were glyoxylated, electrophoresed in a 1% agarose gel and transferred to Nytran (Schleicher and Schuell, Keene, New Hampshire) as described (Sambrook et al., 1989). Hybridizations were performed with the *Kn1* cDNA (Vollbrecht et al., 1991) at 42ºC in 5×Denhardts, 5×SSPE, 100 mg/ml salmon sperm DNA, 2% SDS, and 50% formamide. Filters were washed in 1×SSPE, 0.5% SDS for 30 minutes at room temperature and in 0.2×SSPE, 0.5% SDS at 68ºC twice for 60 minutes. Following autoradiography, filters were stripped and re-probed with a ribosomal probe to assess RNA quality and quantity.

Protein production in E. coli

Full-length KN1 protein was produced in *E. coli* with a T7 polymerase-driven overexpression system (Rosenberg et al., 1987). A plasmid in which the *Kn1* cDNA is fused downstream from a T7 promoter was constructed in two steps. First, a near full-length *Kn1* cDNA (Vollbrecht et al., 1991) with *Eco*RI adaptors was ligated into pPO9, a derivative of pET3C (Rottman, et al., 1991), to create pBVi3. In pBVi3, the *Kn1* open reading frame is out-of-frame with the bacterial translation initiation codon; it was used as a negative control for various experiments as described later. In the second step, the translation initiation codon and 11 amino acid untranslated leader derived from pET3 were removed along with the untranslated leader from the *Kn1* cDNA as follows: pBVi3 was partially digested with *Nco*I, digested with *Nde*I to completion, treated with T4 DNA polymerase and then blunt end ligated to create pBVKN1. Translation of *Kn1* sequences transcribed from pBVKN1 is thus initiated from the putative *Kn1* translation initiation codon. Overexpression from pBVi3 and pBVKN1 was induced as described (Studier and Moffatt, 1986).

Antibody production and purification

KN1 protein produced by induced *E. coli* was found to be highly insoluble. For immunization, insoluble material was purified as previously described (Nagai and Thogersen, 1987). Rabbits were immunized subcutaneously with 0.5 mg of this insoluble material suspended in adjuvant (MPL + TDM + CWS emulsion, RIBI ImmunoChem Research, Hamilton, Montana), and subsequently boosted in an identical manner three times at 3-4 week intervals. Serum was collected 7-10 days after each boost and tested for the titre of antibodies reactive to the 42×10^3 M_r immunizing antigen.

For preparation of an affinity column, the washed, insoluble material described above was solubilized as described (Hoey and Levine, 1988). Concentrations of soluble protein were determined using a Bio-Rad protein determination kit (Richmond, California) with bovine serum albumin (BSA; Sigma, St. Louis, Missouri) as a standard. Soluble protein was coupled to CNBr-activated Sepharose (Pharmacia LKB Biotechnology, Piscataway, New Jersey) according to the manufacturer's instructions. A negative selection column was prepared similarly, coupling the soluble lysate from control *E. coli* strain pBVi3 to sepharose. Antibodies were affinity purified and quantitated as described (Harlow and Lane, 1988). Approximately 50% as much immunoglobulin bound to the affinity column and passed through the negative selection column per ml of preimmune serum compared to immune serum. However, when used at immunoglobulin concentrations equal to that of affinity-purified immune serum, this mock affinity-purified preimmune serum had undetectable binding activity on western blots (not shown) and tissue sections (Figs 3, 5). *Kn1* is a member of a family of homeobox genes in maize (Vollbrecht et al., 1991). The full-length protein used for immunization included the homeobox region that is similar in other members of this family. However, the possibility that the anti-KN1 antibody described here cross-reacts significantly with related homeodomain proteins of the same size is considered unlikely for the following reason. Two monoclonal antibodies have recently been obtained that recognize different epitopes on the full-length KN1 protein, as determined by epitope mapping (L.G.S., unpublished observations). Both of these monoclonals show the same pattern of labelling in tissue sections and western blot experiments as that presented for the polyclonal anti-KN1 antibody. It is very unlikely that both monoclonal antibodies and the polyclonal antibody would recognize epitopes shared by related homeodomain proteins.

Protein extraction and western blotting

Protein extracts from various maize tissues were made by freezing tissues in liquid nitrogen and grinding to a fine powder in a mortar and pestle. Powdered tissues were ground further in the presence of extraction buffer (100 mM Tris pH 7.5, 10% sucrose, 5 mM EDTA, 5 mM EGTA) with a cocktail of proteinase inhibitors (1 mM PMSF, 10 μg/ml of each leupeptin, aprotinin, antipain, benzamidine-HCl and pepstatin, all obtained from Sigma, St. Louis, Missouri) at a ratio of 0.5-2.0 ml/g tissue. Extracts were filtered through glass wool, and soluble protein concentrations determined with the Bio-Rad kit. For SDS-PAGE (Ausubel et al., 1991), extracts were mixed 1:1 with 2×SDS loading buffer, boiled for 5 minutes, and centrifuged for 5 minutes at 16,000 ***g*** to pellet insoluble material. Bacterial pellets were suspended directly in 2× SDS loading buffer and boiled to extract proteins. Following electrophoresis in 10% acrylamide-SDS gels, proteins were electroblotted to Immobilon P membranes (Millipore, South San Francisco, California). Western blot analysis was carried out as described (Ausubel et al., 1991), using affinity-purified antibody at a concentration of 100 ng/ml, and visualizing antibody binding with HRP-conjugated anti-rabbit immunoglobulin and chemiluminescent detection reagents (Amersham, Arlington Heights, Illinois).

BMS transformation

The 1643 bp *Kn1* cDNA (Vollbrecht et al., 1991) was cloned into pC53/ALS (a gift from M. Fromm, Monsanto, St. Louis, Missouri; described in Fromm et al., 1990). This construct contains both the *Kn1* cDNA and the acetolactate synthase gene, which encodes chlorsulfuron resistance, in a 3′ position relative to the Cauliflower Mosaic Virus 35S promoter and the maize alcohol dehydrogenase intron 1. Suspension cultured Black Mexican Sweetcorn cells were bombarded with DNA-coated 1.0 μm tungsten particles using the Biolistic bombardment device (Dupont Co., Wilmington, Delaware) and subsequently cultured in the presence of chlorsulfuron as described (Klein et al., 1987, 1988, 1989). 6-8 weeks after bombardment, chlorsulfuron-resistant colonies were selected and subsequently maintained in callus cultures under continuous chlorsulfuron selection.

Immunohistochemistry

For immunohistochemistry, maize tissues were fixed in fresh 4% paraformaldehyde, 100 mM $NaPO_4$ pH 7.3, 1% DMSO at 4°C with end-over-end rotation for 10-16 hours, rinsed twice for one hour in 100 mM $NaPO_4$ pH 7.3, and dehydrated in an ethanol series. Tissues were then infiltrated and embedded in Steedman's Wax (a mixture of PEG 400 distearate and 1-hexadecanol, Aldrich Chemicals, Milwaukee, Wisconsin) as described (Brown et al., 1989). Ribbons of 8 μm sections were cut from Steedman's Wax blocks in an Tissue Tek II cryostat at 4°C, floated on egg-albumin-coated slides flooded with water, drained and allowed to dry at room temperature for several hours. Sections were dewaxed for 30 minutes in 100% ethanol, and rehydrated in a graded ethanol-water series. Alternatively, tissues were infiltrated and embedded in Paraplast Plus (Monoject Scientific, St. Louis, Missouri) by infiltrating with tertiary butyl alcohol (TBA) via a graded series of ethanol/TBA mixtures, and then incubating in each of the following mixtures for at least 4 hours at 60°C: 25% paraffin/75% TBA, 50% paraffin/50% TBA, 75% paraffin/25% TBA, 100% paraffin, 100% paraffin. Ribbons of 8 μm sections were cut from paraffin blocks on a rotary microtome, floated on poly-L-lysine-coated slides flooded with heated water, drained and allowed to dry for several hours at 37°C. Sections were de-waxed in Histoclear (National Diagnostics, Manville, New Jersey), transferred to 100% ethanol, and rehydrated in a graded ethanol-water series. Dewaxed, rehydrated paraffin sections were treated for 10 minutes at room temperature with proteinase K (Sigma, St. Louis, Missouri) at 100 μg/ml in phosphate-buffered saline (PBS, 150 mM NaCl, 20 mM $NaPO_4$ pH 7.2). The remaining steps were identical for Steedman's Wax and paraffin sections. Slides were incubated in PBS with 1 mg/ml BSA (PBS/BSA) for 30 minutes, then with affinity-purified antibodies diluted to 1 μg/ml in PBS with 1 mg/ml BSA for at least 2 hours. Following two 15 minute rinses in PBS/BSA, slides were incubated in 5 nm gold-conjugated anti-rabbit IgG (Amersham, Arlington Heights, Illinois) diluted 1:40 in PBS/BSA for one hour, and rinsed again as before. Caltag (South San Francisco, CA) silver enhancement reagents were then used according to manufacturer's instructions. Finally, slides were counterstained with basic fuschin diluted 1:20 with water from a 1% stock in 50% ethanol for 30 seconds to 10 minutes, dehydrated in a graded ethanol series, incubated twice for 10 minutes in Histoclear, and mounted permanently with Merckoglas (E. Merck, Darmstadt, Germany). Slides were examined on a Zeiss Universal Electronics Microscope and photographed using Kodak Ektachrome 160T color slide film.

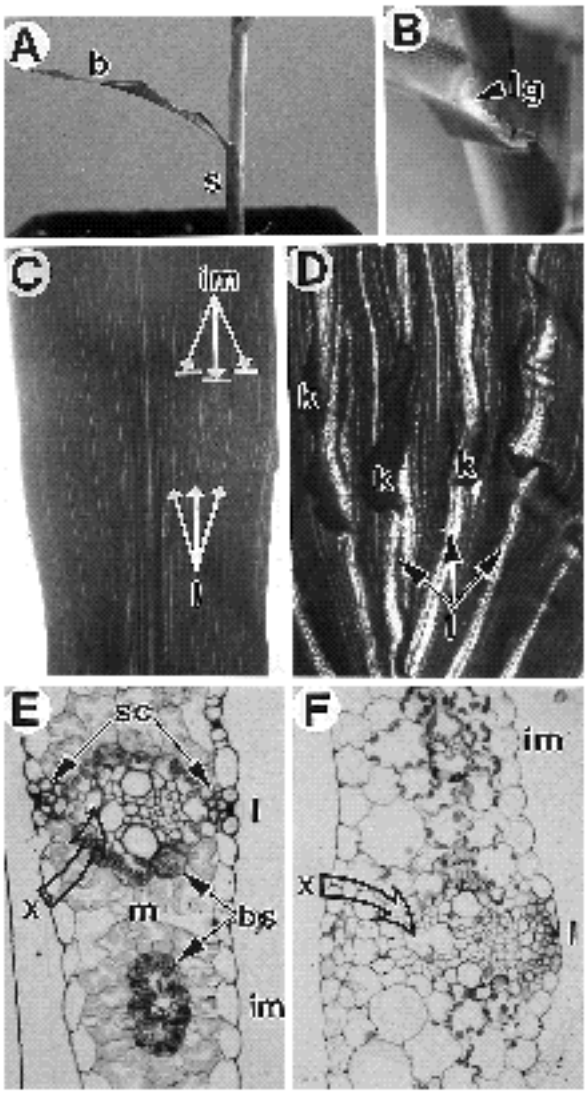

Fig. 1. The morphology of normal and *Kn1-N2* maize leaves. (A) Sheath (s) and blade (b) domains of a maize seedling leaf. (B) Ligule (lg) develops at the boundary between sheath and blade. (C) Wild-type seedling leaf blade, showing the position of lateral (l) and intermediate (im) veins. (D) Seedling leaf blade from a *Kn1-N2* homozygous plant, showing cleared lateral veins (l) and occasional knots (k). (E) Transverse section of a wild-type maize seedling leaf blade, showing morphology of lateral (l) and intermediate (im) veins, each with a bundle sheath (bs), and surrounded by mesophyll cells (m). The xylem pole of the lateral vein is indicated (x); adjacent to both xylem and phloem poles of the lateral are clusters of sclerenchyma cells (sc). Magnification × 140. (F), Transverse section of a *Kn1-N2* homozygous seedling leaf blade through a cleared lateral vein (l) and neighboring intermediate vein (im); the xylem pole of the lateral vein is indicated (x). Magnification × 100).

Results

The Knotted-1 *mutant phenotype*

The maize leaf is divided into two main parts, the sheath and blade, which are separated by a fringe of epidermally derived tissue called the ligule (Fig. 1A, B). The vasculature of the leaf consists of parallel veins; each large "lateral" vein is separated from the next by several smaller "intermediate" veins (Fig. 1C; Sharman, 1942). The normal morphology of lateral and intermediate veins is shown in transverse section in Fig. 1E. Each vein is encircled by a bundle sheath. Both bundle sheath and neighboring mesophyll cells are photosynthetic, but their chloroplasts are morphologically distinct. A cluster of sclerenchyma cells is continuous with the bundle sheath at both poles of lateral, but not intermediate veins. The alterations caused by *Knotted-1* mutations are primarily focused on lateral veins (Gelinas et al., 1969). In homozygous *Kn1-N2* leaf blades, the differentiation of cells around lateral veins is consistently altered. The bundle sheath fails to develop, as does the schlerenchyma normally adjacent to the xylem pole. Lateral veins are instead surrounded by large cells containing few chloroplasts (Fig. 1F; Freeling and Hake, 1985). Macroscopically, these alterations are visible as a wide strip

of unpigmented tissue flanking each lateral vein ("vein clearing"; Fig. 1D). In addition, sporadic foci of extra cell division and expansion along lateral veins produce finger-like protrusions called "knots", which involve all cell layers of the leaf (Fig. 1D; Gelinas et al., 1969; Freeling and Hake, 1985; Sinha and Hake, 1992). *Knotted-1* mutations also alter the development of the ligule, causing displacement of ligule into the blade, and ectopic ligule formation within the blade over lateral veins (Gelinas et al., 1969; Freeling and Hake, 1985).

Kn1 *transcription in wild-type and mutant plants*

A single 1.6 kb mRNA was detected by the *Kn1* cDNA probe in poly (A)-enriched RNA isolated from mature wild-type seedling leaves (Fig. 2A, lane 1), but was undetectable in total RNA isolated from the same tissue (lane 2). Surprisingly, this mRNA is relatively abundant in total RNA isolated from wild-type seedling shoot apices (lane 4). High levels of *Kn1* mRNA are also found in inflorescence primordia from wild-type plants (lanes 6 and 7). Shoot apex RNA isolated from *Kn1-N2* mutant seedlings also contains a single 1.6 kb mRNA of approximately the same abundance as wild-type (lane 5). However, mature seedling leaves from *Kn1-N2* mutant plants accumulate significantly higher levels of *Kn1* mRNA than their wild-type counterparts (lane 3 vs. lane 2). Increased levels of the 1.6 kb *Kn1* mRNA within mutant leaves have also been observed for several other alleles, and in no case have *Kn1* transcripts of altered size been observed (B.G., B.V. and S.H., unpublished observations). These observations support the conclusion that the alterations of non-coding sequence found in dominant *Kn1* mutations change the regulation of *Kn1* transcription without altering the gene product.

KN1 protein in wild-type and mutant plants

To extend the analysis of *Kn1* expression to the protein level, a full-length KN1 protein was overexpressed in *E. coli* using the T7 expression system (Rosenberg et al., 1987). Insoluble KN1 protein was isolated from *E.coli* and used to immunize rabbits. Antisera from these rabbits were affinity purified on a column of solubilized KN1 protein coupled to sepharose. Fig. 2B shows that this affinity-purified rabbit antibody recognizes the M_r 42×10^3 protein in *E. coli* extracts that was used for immunization and affinity purification (lane 1), and has no detectable reactivity to proteins from a control *E. coli* strain that had the *Kn1* cDNA inserted in the T7 expression vector out of frame (lane 2). The affinity-purified antibody also recognizes a single protein of identical apparent M_r in extracts of wild-type vegetative shoot apices and inflorescence primordia (lanes 3-5). No protein is detected in an equally loaded sample of wild-type mature seedling leaf extract (lane 6). Very low levels

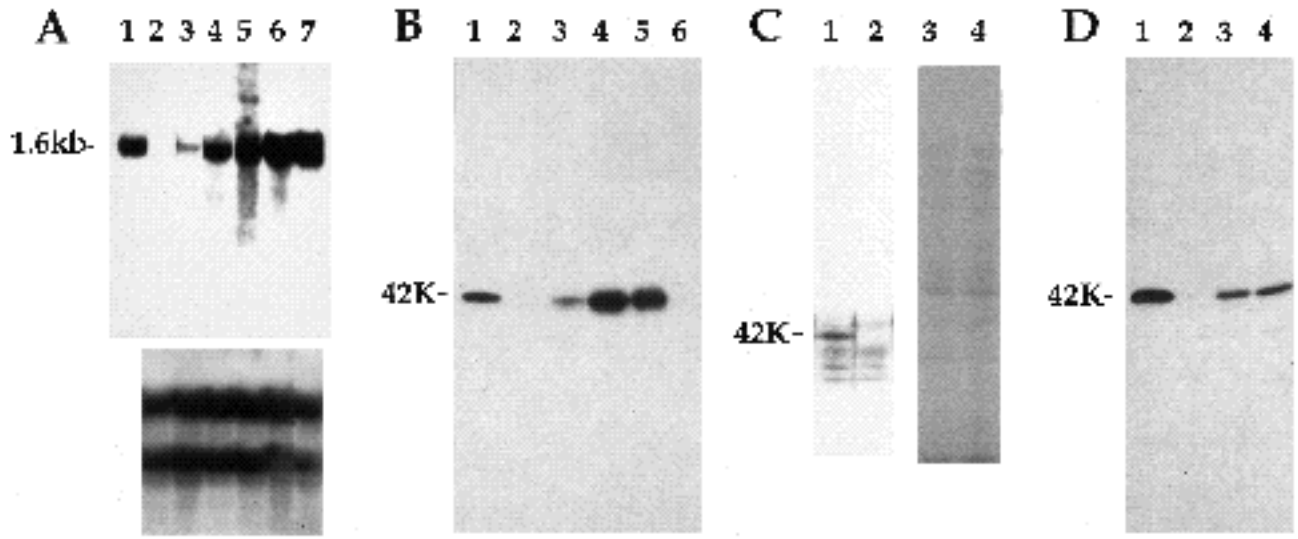

Fig. 2. Northern and western blot analysis of *Kn1* expression. (A) Northern blot probed with *Kn1* cDNA (upper panel), and with a ribosomal probe (lower panel) to verify equal RNA loading. Lane 1 contains 2 μg poly (A)-enriched RNA from mature wild-type seedling leaves; lanes 2-7 each contain 10 μg of total RNA from the following tissues, 2: mature wild-type seedling leaves; 3: mature *Kn1-N2* seedling leaves; 4: wild-type shoot apices; 5: *Kn1-N2* shoot apices; 6: wild-type ear primordia, and 7: wild-type tassel primordia. (B-D) Western blots probed with affinity-purified anti-KN1 antibody. (B) 1: bacterial lysate from induced *E. coli* strain pBVKN1, containing approximately 10 ng of KN1 protein; 2: equal loading of bacterial lysate from induced negative control *E. coli* strain pBVi3; 3: 80 μg protein from wild-type seedling shoot apices; 4: 80 μg protein from wild-type ear primordia; 5: 80 μg protein from wild-type tassel primordia; 6: 80 μg protein from wild-type mature seedling leaves. (C) 1: 100 μg protein from immature (less than fully green) *Kn1-N2* seedling leaves; 2: 100 μg protein from immature wild-type sibling leaves; 3 and 4 are the same as 1 and 2, respectively, stained with Ponceau Red prior to blocking the filter to show approximately equal protein loading. Though anti-KN1 antibody reacts weakly in western blots with other leaf proteins in addition to the M_r 42×10^3 protein, these other proteins are apparently not detectable in the immunolocalization experiments that follow, since they are equally abundant in mutant and wild-type leaf extracts, whereas labelling signals are observed only in mutant leaves. A quantitative increase in the M_r 42×10^3 protein is the only difference between mutant and wild-type leaf extracts detectable by western blotting. (D) 1: same as lane 1 in B; 2: 50 μg protein extracted from BMS cells; 3: 50 μg protein extracted from BMS-*Kn1* cDNA transformant 1; 4: 50 μg protein extracted from BMS *Kn1* cDNA transformant 2.

of this M_r 42×10^3 protein can be detected by the antibody in wild-type leaf extracts under more sensitive detection conditions, however (data not shown). Fig. 2C shows that a M_r 42×10^3 protein is also detected by the antibody in extracts of immature *Kn1-N2* mutant seedling leaf tissue (lane 1), but not in immature seedling leaf extracts from wild-type siblings (lane 2). The mutant leaf extract contained at least 5-fold more of the M_r 42×10^3 protein than wild-type. The antibody to *E.coli*-synthesized KN1 protein thus recognizes a protein of the M_r expected for KN1 in extracts of wild-type plant tissues whose distribution closely parallels that of *Kn1* mRNA, and detects differences in the levels of this protein in mutant and wild-type leaf tissues.

Additional evidence that this protein is the product of the *Kn1* gene was obtained in a separate experiment. Extracts from a line of undifferentiated maize tissue culture cells derived from Black Mexican Sweetcorn (BMS) contain undetectable levels of protein recognized by the antibody (Fig. 2D, lane 2). BMS cells were stably transformed with a construct driving expression of the *Kn1* cDNA from the Cauliflower mosaic virus 35S promoter. Fig. 2D shows that two independent transformants produced high levels of the M_r 42×10^3 protein recognized by the antibody (lanes 3 and 4).

Pattern of Kn1 *expression in wild-type seedlings*

To determine the cellular localization and tissue distribution of the KN1 protein in wild-type maize seedlings, tissue sections were labelled with the affinity-purified antibody, and antibody binding was visualized with silver-enhanced immunogold. Fig. 3A, a median longitudinal section through the vegetative shoot apex of a 2-3 week old maize seedling, illustrates that the anti-KN1 antibody labels nuclei throughout the apical meristem as well as the underlying ground meristem, which gives rise to the non-vascular ground tissue of the stem. No antibody binding was seen in sections labelled with mock affinity-purified pre-immune serum as a negative control (Fig. 3B; see Materials and methods). The nuclear localization of KN1 protein was expected due to the presence of a homeodomain within the protein sequence (Vollbrecht et al., 1991), and supports the hypothesis that KN1 functions as a transcriptional regulator. The boundaries of *Kn1* expression within the shoot apex correspond exactly to the point of insertion of each leaf primordium. No antibody binding was detected within the leaf primordia, including the newly initiated leaf primordium 1 indicated. In fact, a group of unlabelled nuclei can be seen on the flank of the apical meristem in the position where the next leaf will be initiated (P_0). At least 100 unlabelled nuclei were counted in serial sections through this region, a number that is within the range estimated for leaf founder cells by clonal analysis, 100-200 (Poethig, 1984a, b). No antibody binding was detectable within the root tip, including the root apical meristem (Fig. 3C). The absence of immunohistochemically detectable KN1 protein in root tips and leaf primordia indicate that *Kn1* is not expressed by all dividing cells, or all apical meristem cells. In summary, KN1 is found in nuclei of the vegetative shoot apical meristem and subapical ground meristem, and appears to be absent from leaf founder cells on the flank of the meristem just prior to the emergence of a leaf primordium.

Within the immature stem, *Kn1* is also expressed within some developing vascular bundles. The interpretation of these results is aided by a schematic diagram (Fig. 3D) illustrating the arrangement and development of leaf traces (the continuation of leaf veins within the stem) in this region. The first bundles to vascularize the leaf are the laterals, which are initiated within the apical meristem in close association with the corresponding leaf. They then spread upward within the leaf primordium as it grows, and downward in the stem where they form connections with the traces of older leaves (Sharman, 1942). The initiation of these veins within the meristem is accompanied by the downregulation of *Kn1*. This is illustrated in the transverse section shown in Fig. 3E through the shoot apex just below the level of the 4th leaf from the apex, in which round clusters of unlabelled provascular nuclei belonging to the lateral traces of leaves 1-4 can be seen surrounded by labelled ground meristem nuclei. Provascular lateral traces with undetectable antibody labelling can also be seen in longitudinal sections through the shoot apex (Figs 3A, 5A). The subsequent development of lateral traces can be followed by examining transverse sections of successively lower levels of the immature stem, where they can be identified by virtue of their central position within the stem. As lateral vein traces develop, the presence of a closely associated ring of *Kn1*-expressing cells becomes discernable, shown in Fig. 3F just below the level of the 9th leaf from the apex. Cells in this ring will ultimately differentiate into sclerenchyma, after the stem has fully elongated (Esau, 1943). No KN1 protein is detectable in the nuclei belonging to the provascular and parenchymal cells in the interior of these vascular bundles (Fig. 3F). Thus, *Kn1* is downregulated as lateral veins are initiated within the shoot apical meristem, and as these veins develop within the stem, expression of *Kn1* is largely restricted to a ring of cells surrounding the vein.

Kn1 is expressed more extensively in vascular bundles that develop later at the periphery of the immature stem, which by their position appear to include the traces of intermediate veins. Intermediate veins are initiated within the 5th leaf from the apex, and spread downward from the tip of the leaf to the base, subsequently entering and spreading downward through the periphery of the immature stem to form connections with other veins (Sharman, 1942; Fig. 3D). The expression of *Kn1* in a peripherally located provascular trace from the 7th leaf below the apex is illustrated in Fig. 3G. Unlike the provascular lateral bundles illustrated in Figs 3A and 5A, this provascular bundle undergoes a transition in its expression of *Kn1* as it enters the stem (off within the leaf and on within the stem). Fig. 3H also illustrates the expression of *Kn1* in provascular bundles that develop late in the peripheral region of the immature stem (compare provascular bundles in Fig. 3H with Fig. 3E). As these peripheral vascular bundles develop, they often express *Kn1* extensively throughout the interior of the vascular bundle in provascular and parenchymal cells, as shown in Fig. 3I and J. Compare the pattern of *Kn1* expression in these peripheral bundles to that in the centrally located bundles shown in Fig. 3F at a compara-

ble stage of development (one or two protoxylem elements). As the development of stem vascular bundles proceeds, *Kn1* continues to be expressed in nucleated cells around the xylem and phloem poles (Fig. 3K, L). Significantly, however, KN1 is consistently absent from terminally differentiated cells within the vascular bundles of the stem. As illustrated in Fig. 3I, J, K and L, the nuclei of differentiated phloem companion cells and immature xylem elements are unlabelled. In summary, the pattern of *Kn1* expression in developing vascular bundles of the stem correlates with their position and the timing of their development. Vascular bundles that are initiated late at the periphery of the stem express *Kn1* more extensively than those that are initiated early in the central region of the stem. KN1 protein gradually disappears from both vascular bundles and ground tissue cells as internodes elongate (data not shown).

Patterns of Kn1 *expression in wild-type floral shoots*

Since northern and western blots indicated that *Kn1* is strongly expressed in inflorescence primordia (Fig. 2), we examined the patterns of *Kn1* expression during floral development. In Fig. 4, stages of maize inflorescence development are illustrated diagrammatically (after Postlethwaite and Nelson, 1964). After initiating a full complement of leaves, the vegetative apical meristem elongates to form an infloresence meristem (Fig. 4A). This apical inflorescence meristem will give rise to a male inflorescence, the tassel. Female inflorescences develop from axillary buds, which are meristems with the same potential as the apical meristem (illustrated in Fig. 3D). The inflorescence meristems initiate branch primordia (Fig. 4B), which then subdivide into pairs of spikelet primordia (Fig. 4C). The meristem of each spikelet primordium then initiates a pair of glumes, leaf-like organs that will enclose the entire spikelet (Fig. 4D). After subdividing again into upper and lower floret meristems (Fig. 4E), the remaining organs are initiated, finishing with stamen, pistil and ovule primordia (Fig. 4F-I). The floral apical meristem itself is terminated in the production of the ovule primordium. These initial events proceed similarly in ear and tassel primordia, which subsequently undergo selective stamen and pistil abortion, respectively, to generate the unisexual inflorescences of maize (Cheng et al., 1983). Though the ontogeny of the vasculature of floral shoots has not been extensively characterized, its overall organization is comparable to that of the vegetative shoot (Laubengayer, 1949), and probably develops similarly.

Fig. 5A shows that *Kn1* is expressed throughout the apical meristem as it elongates to form an inflorescence

Fig. 3. Immunolocalization of KN1 protein in wild-type maize seedlings. All tissue sections except B are labelled with the affinity-purified anti-KN1 antibody. Sections were counterstained with basic fuschin, so nuclei not labelled with silver grains are stained pink. (A) Median longitudinal section through the vegetative shoot apex of a 2-3 week old maize seedling, indicating the youngest 3 leaf primordia (numbered), the P_0 region of the shoot apical meristem, provascular bundles (pr), and ground meristem cells (gm). (B) Equivalent section to A, but labelled with preimmune antibody as a negative control. (C) Median longitudinal section through the root tip, including the root apical meristem (ram). (D) Diagrammatic representation of the vegetative shoot apex, showing the shoot apical meristem (sam), axillary buds (ab), and leaf primordia (numbered) with their associated veins. Provascular bundles are indicated by broken lines; veins with differentiated vascular elements are indicated by solid lines. Lateral veins are shown in red, intermediate veins in blue. For simplicity, only one vein of each type is shown for each leaf. Lateral veins are initiated first, and their traces occupy the central region of the stem. Intermediate veins are initiated later within the leaf and spread downward into the stem, where their traces remain in the peripheral region of the stem. The level of section in panels E-J is indicated by arrows. Based on data from Sharman, (1942) and Kumazawa, (1961), which describe these processes in more detail. (E) Transverse section through the shoot apex just below the point of insertion of the 4th leaf from the apex, showing provascular bundles (pr) and ground meristem cells (gm). (F) Transverse section through the central region of the immature stem at the level of insertion of the 9th leaf from the apex showing the traces of lateral veins surrounded by rings of *Kn1*-expressing cells. (G) Longitudinal section at the point of insertion of the 7th leaf from the apex, where a provascular strand (pr) passes from the leaf (l) into the stem (s) and undergoes a transition in its expression of *Kn1*. (H) Transverse section through the peripheral region of the immature stem at the same level as F, showing bundles that are mainly provascular (pr), which develop late in the periphery of the stem; the thick arrow points toward the center of the stem. (I, J) Transverse sections at the level of insertion of the 10th leaf from the apex, showing immature peripherally located vascular bundles with differentiated vascular elements; xylem (x) and phloem (p) areas are indicated by arrows. (K, L) More advanced immature vascular bundles of the stem within a young internode between the points of insertion of the 11th and 12th leaves from the apex; xylem (x) and phloem (p) areas of the bundle are indicated. Bar = 100 μm.

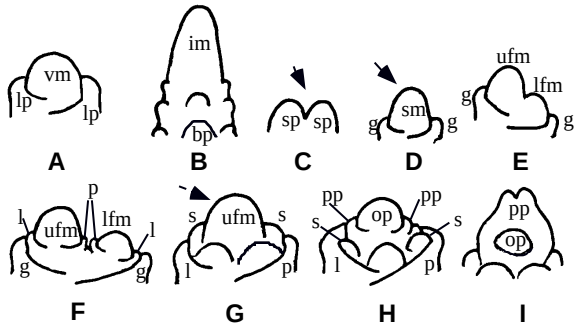

Fig. 4. Diagrammatic representation of maize inflorescence development, re-drawn from Postlethwaite and Nelson, 1964. (A) The vegetative meristem (vm) initiates leaf primordia (lp). (B) After initiating a full complement of leaves, the vegetative meristem becomes an elongating inflorescence meristem (im) that initiates branch primordia (bp); (C) branch primordia subdivide to form pairs of spikelet primordia (sp); (D) spikelet meristems initiate a pair of bracts called glumes (g); (E) spikelet meristems then subdivide to form an upper and a lower floret meristem (ufm, lfm); (F) both upper and lower floret meristems initiate another pair of bracts, the lemma (l) and palea (p); (G) floret meristems then initiate stamen primordia (s); (H) floret meristems finally initiate pistil primordia (pp) and are terminated in the production of an ovule primordium (op). (I) The pistil primordium (pp) forms a hood-like structure around the ovule primordium (op) that will elongate to form a silk.

meristem, and is also strongly expressed in each axillary bud of the shoot. Expression of *Kn1* at a later stage of tassel development, midway through the initiation of floral organs, is shown at low magnification in Fig. 5B. *Kn1* is strongly expressed in each lateral floral meristem, and is also present in ground meristem and immature vascular bundles within the floral shoot axis. No antibody binding is detected in a similar section labelled with mock affinity-purified preimmune serum as a negative control (Fig. 5C). As expected, since ear and tassel development have not yet substantially diverged at this stage, the pattern of *Kn1* expression at a comparable stage of ear development is similar (data not shown). Fig. 5D-G show that *Kn1* is downregulated as floral organs are initiated by floral meristems, as described previously for the initiation of leaves by the vegetative apical meristem. In Fig. 5D, spikelet meristems can be seen along the length of an ear primordium that strongly express *Kn1* in all cell layers. In Fig. 5E, KN1 protein is present in the nuclei of the spikelet meristem, but is undetectable in nuclei of newly initiated glume primordia. Fig. 5F shows that *Kn1* is downregulated as each subsequent set of floral organs is initiated, but continues to be expressed in the floret meristem itself. Finally, as shown in Fig. 5G, apical expression of *Kn1* disappears as the floret apical meristem is terminated in the production of the ovule primordium, such that *Kn1* expression becomes restricted to the internal, ground meristem of the immature floret. Fig. 5H shows that in a transverse section through the axis of a tassel primordium at a comparable stage to that in Fig. 5B, the patterns of *Kn1* expression in immature vascular bundles are similar to those analyzed in more detail for the vegetative shoot. In summary, the expression of *Kn1* in developing floral shoots is analogous in every respect to that described for vegetative shoots. It is present in nuclei of floral apical meristems and disappears as floral organs are initiated, and is also expressed within the axis of the floral shoot in ground meristem cells and immature vascular bundles. This suggests that *Kn1* functions similarly during the development of vegetative and floral shoots, rather than playing a unique role in floral development.

Ectopic expression of Kn1 *in mutant leaves*

To investigate the basis of the developmental alterations in *Kn1* mutant plants, we compared the patterns of *Kn1* expression in mutant plants with their wild-type siblings. Northern blot analysis showed that *Kn1* mRNA is equally abundant in total RNA isolated from wild-type and *Kn1-N2* mutant vegetative shoot apices (Fig. 2A). Likewise, examination of KN1 protein localization in tissue sections of vegetative shoot apices revealed no obvious differences between normal and mutant plants (data not shown). Despite the presence of low levels of *Kn1* mRNA and KN1 protein detectable in wild-type leaves by northern and western blotting (Fig. 2), KN1 protein is undetectable immunohistochemically within wild-type leaves at all stages examined. The KN1 protein in wild-type leaves must therefore be present at levels too low to detect with the techniques used here, or is present in rare cells that were missed. Since northern and western blot data indicated that there was a significant increase in *Kn1* expression in mutant leaves (Fig. 2), we sought to localize KN1 protein in developing leaves of homozygous *Kn1-N2* seedlings.

Ectopically expressed KN1 protein is detectable initially in nuclei of immature lateral veins, the veins most severely affected by *Knotted-1* mutations (Fig. 6A). A comparable transverse section through wild-type sibling leaves shows no detectable antibody binding (Fig. 6B). The earliest stage of leaf development at which ectopic expression of *Kn1* was consistently observed was in leaves that were the 5th from the apex, in lateral veins that had at least one differentiated protoxylem element, and were separated by several provascular intermediate bundles. The patterns of ectopic *Kn1* expression at this stage of lateral vein development in mutant leaf blades are comparable to those observed in vascular bundles of wild-type vegetative and floral shoot axes at a comparable stage. Though there was some variability in the strength of the signal and the combination of cells labelled, virtually every lateral was found to be affected (Fig. 6A). An example of the most common initial pattern is shown at higher magnification in Fig. 6C. Nuclei of cells encircling the immature vein (precursors of bundle sheath cells) as well as within it (provascular and parenchymal cells) express *Kn1*, primarily in the half of the bundle that contains the xylem pole. This observation is significant, because it is the cells within and around this half of the lateral vein that are most severely affected by the *Kn1-N2* mutation, as shown in Fig. 1F compared to Fig. 1E. As mutant leaf blades develop further, *Kn1* continues to be expressed in somewhat variable patterns in and around lateral veins, and is also detected in many immature intermediate veins when their protophloem and protoxylem begin to differentiate (Fig. 6D). Ectopic expression of *Kn1* in immature intermediate veins is therefore a relatively late event in the development of mutant leaf blades. Just prior to the completion of cell expansion and photosynthetic maturation of the leaf blade, persistent ectopic expression of *Kn1* can be seen both within cleared lateral veins (Fig. 6E), and also within young knots that have begun to form by this time (Fig. 6F). No unique pattern of ectopic *Kn1* expression was found associated only with knots that might explain why knots form where they do, though such a correlation may yet be found. Ectopic localization of *Kn1* mRNA within veins of mature *Kn1-N2* leaf blades has previously been described (Sinha, 1990).

Discussion

Although dominant *Knotted-1* mutations alter leaf development, we have found that *Kn1* is not expressed at high levels during the development of wild-type leaves. KN1 is present at high levels in vegetative apical and axillary meristems, but is immunohistochemically undetectable in leaf founder cells on the flank of the apical meristem, and at all stages of wild-type leaf development examined. Similarly within wild-type floral shoots, *Kn1* is expressed in floral apical meristems, but is downregulated as floral organs are initiated and as the apical meristem is finally terminated in the production of the ovule primordium. Within the immature, unelongated axes of vegetative and floral shoots, *Kn1* is also expressed in relatively undifferentiated

cells in ground tissue and developing vascular bundles. As expected from the presence of a homeobox in the *Kn1* gene (Vollbrecht et al., 1991), KN1 protein is found in nuclei, supporting the hypothesis that it functions as a transcriptional regulator.

In *Kn1-N2* plants, neither *Kn1* transcripts nor KN1 protein appear to be altered qualitatively, but levels of *Kn1* mRNA and KN1 protein are significantly increased within leaves. KN1 protein is localized ectopically in lateral veins of mutant leaves from an early stage of leaf development, and subsequently in many intermediate veins as well. The patterns of ectopic expression within developing veins of mutant leaf blades are similar to those observed in the developing veins of wild-type vegetative and floral axes. The regulation of *Kn1* expression thus appears to be subtly altered by the *Kn1-N2* mutation such that *Kn1* is expressed in vascular bundles developing both in the leaf and the stem instead of being restricted to bundles of the stem. The ectopic *Kn1* expression within veins of mutant leaf blades correlates with, and is likely to cause, the alterations in cell fate produced by the *Kn1-N2* mutation. In the severely affected *Kn1-N2* homozygotes analyzed here, all lateral veins are affected by vein clearing as shown in Fig. 1D and, in addition, knots form sporadically along lateral veins as a result of localized additional growth relative to surrounding tissue. We cannot presently explain why ectopic expression in lateral veins would lead consistently to vein clearing and only sporadically to knot formation; the placement of knots may depend on additional factors other than the pattern or the levels of ectopic *Kn1* expression within lateral veins. The effects of *Kn1-N2* on intermediate vein development are less severe, including a reduction in the density of bundle sheath chloroplasts (L.S., unpublished observation), alterations in the distribution of photosynthetic enzymes in bundle sheath and mesophyll cells in the region of a knot (Sinha, 1990), and increased intervein spacing due to extra growth between intermediates in the region of a knot (Fig. 1D). Presumably, ectopic expression of *Kn1* in intermediate veins affects their development only mildly, because it is a relatively late event in the development of the leaf as a whole.

Though ectopic expression of *Kn1* is closely associated with veins in the leaf, the phenotypic effects of *Kn1* mutations spread beyond the veins, altering the differentiation and growth of surrounding cells as well. For example, the differentiation of mesophyll cells within a broad area around the vein is altered in cleared laterals (Fig. 1F), the differentiation of epidermal cells is altered when a ligule forms ectopically over lateral veins, and the formation of knots involves all cell layers of the leaf (Sinha and Hake, 1992). However, the conclusion that ectopic *Kn1* expression is the primary cause of all aspects of the *Kn1-N2* phenotype is strongly supported by previous analysis of genetic mosaics. Knot formation was found to depend on the genotype of internal tissue layers and not on that of the epidermis (Hake and Freeling, 1986). Subsequent analysis showed that the presence of the *Kn1-N2* allele in only the innermost cell layer of the leaf was necessary and sufficient for knot formation and ligule displacement (Sinha and Hake, 1990). The innermost cell layer of the leaf includes the vascular bundles and a single layer of mesophyll cells (Langdale et al., 1989). Taken together, these data argue that *Kn1* alters the fates of leaf cells in which it is ectopically expressed, as well as their neighbors in the mesophyll and epidermis. The mechanism by which the behavior of surrounding cells is affected is unknown, but may simply reflect an inability on the part of individual cells to grow, divide and differentiate completely independently of their neighbors.

The alterations in cell division, growth and differentiation that apparently result from ectopic expression of *Kn1* in the leaf suggest that, in its wild-type context, it functions to regulate these processes as well. We hypothesize that *Kn1* may function to oppose determination, the process by which cells become committed to their developmental fates, manifested initially at the shoot apex in patterns of cell division and growth, and ultimately in cellular differentiation. The downregulation of *Kn1* within a group of cells on the flank of the apical meristem might thus be important for the determination of a lateral organ with developmental potential and growth characteristics distinct from the apical meristem, ie., “determinate” or limited potential and rapid, polarized cell growth and division. Indeed, the timing of *Kn1* downregulation within the vegetative apical meristem just prior to the emergence of a leaf primordium coincides with the time in other angiosperms at which the site of leaf initiation is apparently fixed (Snow and Snow, 1933), and the determinate nature of the leaf is established (Sussex, 1955; Snow and Snow, 1959). At the same time, an increase in the frequency of cell divisions in the plane characteristic of the future leaf also becomes discernable, marking the beginning of a new pattern of growth and cell division that initiates the leaf (Lyndon, 1983). Within the immature axes of vegetative and floral shoots, *Kn1* might similarly oppose the differentiation and rapid, longitudinally polarized growth and division of cells within ground meristem and immature vascular bundles, since KN1 is consistently absent from terminally differentiated cells and disappears gradually as internodes elongate. However, *Kn1* clearly could not by itself be responsible for setting the differentiated state of each cell, since *Kn1* is expressed by some partially differentiated cells, such as provascular cells, as well as completely undifferentiated apical meristem cells. Furthermore, similar partially differentiated cells do not always express *Kn1* in the same way. We therefore suggest that *Kn1* acts co-operatively with other regulators, so that the outcome of *Kn1* expression for any given cell depends on additional influences acting on that cell.

Many aspects of the *Knotted-1* mutant phenotype can be interpreted as a displacement or substitution of sheath-like development in the blade along lateral veins. For example, the formation of ectopic ligule tissue suggests that novel sheath-blade boundaries are formed within *Knotted-1* leaf blades parallel to lateral veins and, indeed, the epidermal cells between the ectopic ligule fringes appear similar to sheath epidermal cells by scanning electron micrography (Becraft and Freeling, 1989). Though these changes apparently result from the ectopic expression of *Kn1* in the leaf blade, we have found no evidence that *Kn1* plays a role in the development of sheath tissues in wild-type plants, but rather have suggested that *Kn1* opposes cell determination. However, in *Kn1-N2* leaf blades, ectopic *Kn1* expression is

restricted to a small group of cells within a primordium that has already progressed considerably along the pathway of leaf development. Consistent with the proposal that the outcome of *Kn1* expression for any given cell depends on additional influences acting on that cell, we suggest that such temporally and spatially restricted expression of *Kn1* within the context of the leaf primordium cannot by itself reverse the determination of those cells as leaf cells. In this context, *Kn1* may simply delay the progression of those cells to their final fates, which are consequently altered. This could explain both the adoption of sheath-like fates and the inappropriate growth leading to knot formation, since the sheath portion of maize leaves completes its cell division and differentiation after the blade (Sharman, 1942; Sylvester et al., 1990).

Dominant developmental mutations caused by ectopic expression of homeobox genes have previously been described in animal systems. For example, dominant mutations in the *Drosophila* homeotic selector genes *Ultrabithorax*, *Antennapedia* and *Deformed*, produced by a variety of changes at the DNA level that alter the regulation of these genes, cause ectopic expression and result in the respecification of body segments (White and Akam, 1985; Cabrera et al., 1985; Frischer et al., 1986; Chadwick et al., 1990). Together with *Knotted-1*, these examples illustrate the point that neomorphic dominant mutations can disrupt the development of tissues where they may not normally function. The alterations produced by ectopic expression can nevertheless provide insight into the function of the wild-type gene product in its normal context. To obtain further evidence pertaining to the role of *Kn1* in development, the *Kn1* cDNA is being expressed ectopically in heterologous host plants, and we are attempting to create loss-of-function alleles of *Kn1*. We hope that these efforts will provide further insights into the mechanisms controlling plant pattern formation.

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Fig. 5. Immunolocalization of KN1 protein during inflorescence development in wild-type plants. All tissue sections except C are labelled with the affinity-purified anti-KN1 antibody, and counterstained with basic fuschin. (A) Median longitudinal section through the shoot apex in transition to floral development, showing the inflorescence meristem (im), provascular lateral traces (pr), and axillary buds (ab). (B) Longitudinal section through a tassel primordium midway through the elaboration of floral organs. (C) Equivalent section to B, but labelled with preimmune antibody as a negative control. (D) Median longitudinal section through the axis of an early ear primordium bearing spikelet meristems. (E) Longitudinal section through ear spikelet meristems with glume primordia (g). (F) Longitudinal section through an ear floret showing the primordia of a stamen (s), palea (p), and glume (g). (G) Longitudinal section through an ear floret terminated by an ovule primordium (op). (H) Transverse section through the axis of a tassel primordium at a comparable stage to that in shown in B, showing immature vascular bundles surrounded by ground meristem cells. Bar = 200 μm.

Fig. 6. Immunolocalization of KN1 protein in homozygous *Kn1-N2* and wild-type sibling leaf blades. All tissue sections are labelled with the affinity-purified anti-KN1 antibody, and counter-stained with basic fuschin. (A) Transverse section through immature, *Kn1-N2* leaf blades; arrows point to antibody-labelled lateral veins. (B) Transverse section through immature, wild-type sibling leaf blades shows no antibody labelling. (C) Transverse section at higher magnification shows an example of the most common pattern of KN1 protein localization at an early stage of lateral vein development in a *Kn1-N2* leaf blade. (D) Transverse section through a more advanced immature *Kn1-N2* leaf blade, showing labelled lateral (l) and intermediate (im) veins. (E) Transverse section through a young *Kn1-N2* leaf blade showing labelled nuclei associated with a lateral vein (l), with its xylem pole marked (x), exhibiting typical “cleared vein” morphology, and flanking intermediate veins (im). (F) Transverse section through a young knot looping out from a *Kn1-N2* leaf blade; labelled nuclei are indicated by arrows. Bar = 100 μm.