

## Loss-of-function mutations in the maize homeobox gene, *knotted1*, are defective in shoot meristem maintenance

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### SUMMARY

The product of the maize homeobox gene, *knotted1* (*kn1*), localizes to the nuclei of cells in shoot meristems, but is absent from portions of the meristem where leaf primordia or floral organs initiate. Recessive mutant alleles of *kn1* were obtained by screening for loss of the dominant leaf phenotype in maize. Mutant *kn1* alleles carrying nonsense, splicing and frame shift mutations cause severe inflorescence and floral defects. Mutant tassels produce fewer branches and spikelets. Ears are often absent, and when

present, are small with few spikelets. In addition, extra carpels form in female florets and ovule tissue proliferates abnormally. Less frequently, extra leaves form in the axils of vegetative leaves. These mutations reveal a role for *kn1* in meristem maintenance, particularly as it affects branching and lateral organ formation.

Key words: meristem, homeobox, *knotted1*, maize

### INTRODUCTION

The majority of the higher plant body is elaborated from events occurring at the growing tips. The entire above ground portion of the typical plant derives from the shoot apical meristem (SAM) while the below ground portion arises from the action of root apical meristems (for review see Steeves and Sussex, 1989). The SAM is indeterminate, capable of producing an unspecified number of lateral organs. It is the site of initial organogenesis, giving rise to determinate lateral organs such as leaves and axillary branches. After floral induction, the SAM forms inflorescence and floral meristems which themselves initiate specialized lateral organs involved in reproduction. The SAM is also self-renewing. The SAM contributes cells to the formation of lateral organ primordia, and these cells are continually replaced; as a result, the meristem itself is maintained during the process of organ initiation. Understanding the developmental events that determine the overall form of a plant (branching, leaf and floral organogenesis, etc.) requires careful examination of events occurring in the meristem.

The maize homeobox gene *knotted1* (*kn1*) has proved to be a useful molecular marker for the SAM (Smith et al., 1992; Jackson et al., 1994). The onset of *kn1* expression during embryogenesis coincides with the first histological features that characterize SAM formation in maize (Smith et al., 1995). *kn1* is expressed not only in the vegetative SAM, but also in axillary meristems, in terminal and lateral inflorescence meristems (tassel and ear, respectively), and in both male and female floral meristems. *kn1* is not expressed in the determinate products of meristems such as leaves and floral organs.

The disappearance of *kn1* from the flanks of the meristem correlates well with the site of initiation of the next organ primordium (Smith et al., 1992; Jackson et al., 1994).

*kn1* is a member of a family of closely related homeobox genes in maize (Vollbrecht et al., 1991). Eight class 1 *kn1*-like homeobox (*knox*) genes have been characterized in maize (Kerstetter et al., 1994). These family members share a high degree of sequence similarity in the homeodomain (HD) and in specific regions outside the HD, and several *knox* genes show overlapping expression patterns in meristems (Jackson et al., 1994; Kerstetter et al., 1994). These observations may indicate that *knox* genes provide partially redundant functions in the shoot meristems of the maize plant. *knox* genes have been reported in related grasses such as rice (Kano-Murakami et al., 1993) and barley (Müller et al., 1995), and in more distantly related dicot species such as *Arabidopsis*, soybean and tomato (Lincoln et al., 1994; Ma et al., 1994; Long et al., 1996; Hareven et al., 1996).

Dominant mutations in the maize *kn1* gene and in *kn1*-like genes have been described (Gelinis et al., 1969; Hake et al., 1989; Müller et al., 1995; Schneeberger et al., 1995). Ectopic expression of KN1 in lateral veins of the maize leaf blade interferes with the normal differentiation of cells around the lateral veins (Smith et al., 1992). Affected cells proliferate to form sporadic outgrowths or 'knots' of tissue and take on characteristics of more basal portions of the leaf such as auricle and sheath (Sinha and Hake, 1994). Expression of the maize *kn1* cDNA in dicot species has profound effects on the shoot. Transgenic tobacco, *Arabidopsis* and tomato plants show retarded growth, reduced apical dominance and perturbed leaf development (Sinha et al., 1993; Lincoln et al., 1994; Hareven

et al., 1996). In tobacco and *Arabidopsis*, leaves are thickened, can be lobed, and, in severe cases, shoots arise on the adaxial leaf surface (Sinha et al., 1993; Chuck et al., 1996). These results indicate that expression of *kn1* in leaves is sufficient to induce ectopic meristem formation in transgenic tobacco and *Arabidopsis* leaves.

In order to determine the normal function of *kn1* in maize, we have isolated recessive alleles of the locus by selecting for the loss of the dominant mutant phenotype. Here we report on the phenotypes of plants carrying loss-of-function alleles of *kn1* and discuss the role of *kn1* in specifying meristem identity in maize development.

## MATERIALS AND METHODS

### Mutagenesis

Linkage of the *Kn1-N* allele to a specific allele of *alcohol dehydrogenase 1* (*adh1-S*) and the presence of a non-autonomous receptor of the *Dotted* (*rDt*) transposable element in the fourth intron (Brown et al., 1989; Hake, 1992) were used to follow the putative loss-of-function alleles in genetic crosses and to control for contaminating pollen. Pollen, collected from plants of the following genotype {*Kn1-N*, *adh1-S*}/*{def(kn1)-O*, *adh1-F6*}, was treated for 30 to 50 minutes in a 0.1% (v/v) emulsion of ethane methyl sulfonic acid (EMS) in mineral oil according to the method of Neuffer (1994) and applied with a brush to silks of the inbred line B73 (Pioneer HiBred International, Inc.). Seeds were collected, planted at high density, and screened as seedlings for individuals without knots. The EMS-induced derivatives no longer expressing the knotted phenotype were self-pollinated (where possible) and the progeny were examined for the absence of knots and the *Adh1-S* and *rDt* markers.

### RNA isolation and blot hybridization

Total RNA was prepared from 4-6 pooled maize seedling meristems (including stem and youngest leaf primordia) as described by Smith et al. (1992). RNA blots containing 10 µg per lane were hybridized as previously described (Smith et al., 1992). Probes specific for *kn1* were derived from the 3' untranslated region of the *kn1* cDNA by PCR using the primers E41 (CCTTCTACATGGACGGCCAC) and E42 (GGATCCATCTGTCAGGTTAC). Blots were stripped and re-probed with a portion of an oat β-tubulin cDNA (Colbert et al., 1990), which detects a single size class of mRNA in maize, to assess RNA quality and quantity.

### Monoclonal antibody preparation

Insoluble, full-length maize KN1 protein produced in *E. coli*, and purified as described (Smith et al., 1992) was mixed 1:1 with 2× reconstituted adjuvant (RIBI Adjuvant System, RIBI Immunochem Research, Inc., Hamilton, MT) to a final volume for injection of not more than 100 µl per mouse. Swiss-Webster mice were injected subcutaneously with 50 µg of the antigen every 2 to 3 weeks until an adequate titer was raised, as assayed by dot immunoblot analyses. The electrofusion protocol and postfusion hybridoma maintenance were as described by Karu (1993). Hybridoma supernatants were transferred to immunological assay plates using a robotic sampling system (Karu et al., 1985). The cell lines were assayed by ELISA and immunoblots, and were tested for specificity on maize tissue sections. Selected hybridomas were subcloned and stored in liquid nitrogen. Crude epitope mapping was performed by testing reactivity of monoclonal antibodies on immunoblots of KN1 protein deletion derivatives produced in *E. coli*. The monoclonal cell line supernatant chosen for this study is specific for a portion of the KN1 protein upstream of the homeodomain or ELK/homeodomain regions of KN1 (D. Jackson and S. Hake, unpublished results).

### Protein isolation and immunodetection

Protein extracts were prepared from individual young maize ears as described by Smith et al. (1992). The soluble protein concentrations were determined with a Bio-Rad kit. For SDS-PAGE (Ausubel et al., 1991), 350 µg of each protein extract was mixed with 2× SDS loading buffer and boiled for 10 minutes. Following electrophoresis in 10% acylamide-SDS gels, proteins were electroblotted to nitrocellulose membranes. Western blot analysis was carried out as described by Ausubel et al. (1991), using mAb crude supernatant at a 1:80 dilution in milk buffer (100 mM Tris pH 7.4, 100 mM NaCl, 2% nonfat dry milk). Bound mAb was visualized with alkaline phosphatase-conjugated anti-mouse immunoglobulin G and colorimetric detection reagents (NBT and BCIP, Gibco-BRL, Gaithersburg, MD). Immobilized proteins on the filter were stained after the western blot was developed to demonstrate equal protein loading.

### Amplification and sequencing

Exon sequences of alleles of *kn1* were PCR amplified using oligonucleotides synthesized on a DNA synthesizer (model 380B; Applied Biosystems, Foster City, CA) from 1 µg of maize genomic DNA, prepared as previously described (Chen and Dellaporta, 1994). PCR products were gel purified and sequenced directly with the *f* mole sequencing kit from Promega (Madison, Wisconsin) using end labeled primers. Primer sequences are as follows:

- 1 (RKn5P) GAGAAGGCATACTCCGAGGCATCC;
- 2 (RKn11A) CCAAACCTTCGCTAGCAGCAGTAGCT;
- 3 (RKn11B) GTAGACATGTGCATCTGGTGTTCGGA;
- 4 (RKn13) CATGCATCGCTGCATGAGGCGACA;
- 5 (R7436) AGTTCAGTAAATGTCTTTGCCGGA;
- 6 (rDt) CAAGGCAGTACTCCAATAGT;
- 7 (E38) TATTAATTTAACCTTATTTAAGG;
- 8 (E31) ATAGGCGCATATAGATAGAGTAGCT;
- 9 (REM52) CGATCGCTGCACCACTACTTCTC;
- 10 (REM53) GGCCTGCCGCTGACCCTGAACACG.

The entire coding region and intron/exon borders were sequenced for each allele.

### Scanning electron microscopy

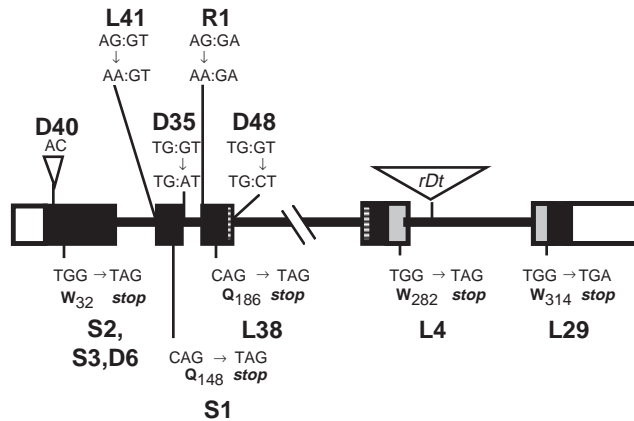
Young ear primordia were fixed in FAA (10% formalin, 5% acetic acid, 45% ethyl alcohol) with 1% Triton X-100, dehydrated through a graded ethanol series, and critical point dried in liquid CO<sub>2</sub>. Mounted specimens were sputter coated with 25 nm of Au/Pd and viewed in an ISI DS130 scanning electron microscope with an accelerating voltage of 10 kV.

## RESULTS

### An insertion, nonsense mutations and splice defects produce loss-of-function alleles of *kn1*

The dominant *Kn1* mutant phenotype results from ectopic *kn1* expression in leaf cells with no disturbance of the normal pattern of expression (Smith et al., 1992). We screened approximately 28,000 *Kn1-N* heterozygous seedlings for loss of the dominant mutant phenotype following chemical mutagenesis (Neuffer, 1994) of pollen carrying the dominant allele. Twelve seedlings showed no signs of the dominant mutant phenotype and were backcrossed for three to six generations into the standard inbred line B73 to eliminate unlinked EMS mutations.

The *Kn1-N* gene structure and EMS mutations are depicted in Fig. 1. The alleles *NL41* and *NR1* both have single base pair transitions (G to A) in the invariant AG dinucleotide on the intron side of the 3' splice acceptor adjacent to exon 2 and 3 respectively (Fig. 1). *ND35* and *ND48* carry single base pair



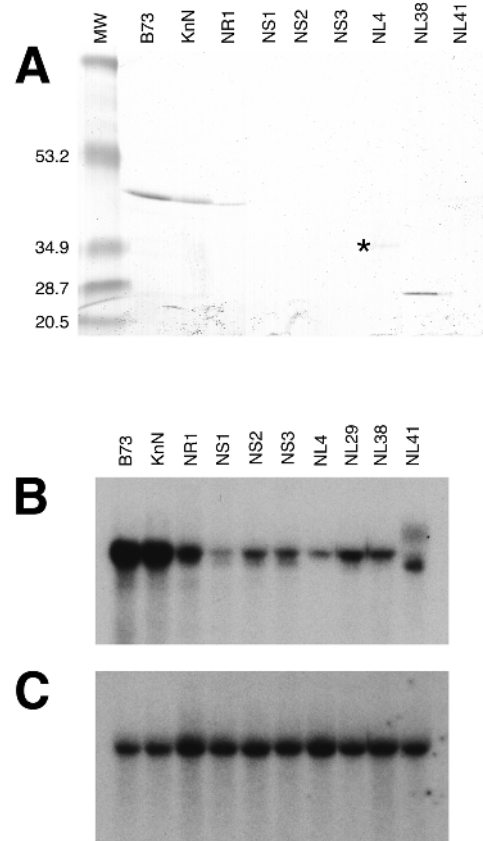
**Fig. 1.** Schematic representation of *kn1* gene structure and EMS derivatives. The *kn1* coding region is interrupted by 4 introns and encodes a 359 amino acid polypeptide (Vollbrecht et al., 1991). The positions of EMS mutations in the gene are shown with allelic designations in boldface. Nucleotide changes are indicated, as are resulting changes in the predicted polypeptide. Subscripts indicate positions of individual amino acids and colons indicate exon/intron junctions. Exons are represented by boxes, introns by lines; the large (>5 kb) third intron has been truncated in this schematic. Filled boxes indicate coding regions, unfilled boxes indicate untranslated regions of the cDNA, stippling indicates the homeobox, and stripes indicate the putative PEST sequence. The triangle indicates the position (and relative size) of the *rDt* transposon in the *Kn1-N* progenitor and all of the EMS derivatives.

changes (G to A and G to C, respectively) in the invariant GT dinucleotide on the intron side of the 5' splice acceptor adjacent to exons 2 and 3. Alleles *NS2*, *NS3*, *ND6*, *NS1*, *NL38*, *NL4* and *NL29* carry stop codons located along the length of the *kn1* coding region as indicated in Fig. 1. *NS2*, *NS3* and *ND6*, each isolated independently, carry identical nucleotide substitutions. In the *ND40* allele, a 2 nucleotide insertion 46 bp downstream of the first ATG causes a reading frame shift.

### Loss-of-function mutants show reduced levels of KN1 protein and RNA

To determine the effects of the new *kn1* alleles on the gene product, expression of KN1 was compared in normal and a subset of the homozygous mutant plants. A monoclonal antibody (mAb), raised against a full length KN1 protein over-expressed in *E. coli*, was used to probe an immunoblot prepared with proteins extracted from individual young ears. Fig. 2A shows that the mAb recognizes a  $42 \times 10^3 M_r$  protein in ears of the standard inbred line B73 and in *Kn1-N* heterozygotes. Full length or nearly full length KN1 is also detected in ear proteins from *NR1* (Fig. 2A), although the levels of protein appear reduced compared to B73 and *Kn1-N*. KN1 protein was not detected in ears of several of the mutants including *NS1*, *NS2*, *NS3* and *NL41* (Fig. 2A). Truncated proteins were detected in *NL38* and *NL4*, each consistent with the size predicted by the position of the nonsense mutation. The *NL38* band, though smaller, appears more intense than the very faint *NL4* band (Fig. 2A). The apparent abundance of *NL38* protein may be related to the position of the stop codon which occurs before a candidate PEST sequence (Vollbrecht et al., 1991).

Total RNA was isolated from shoot meristems of 2-week old

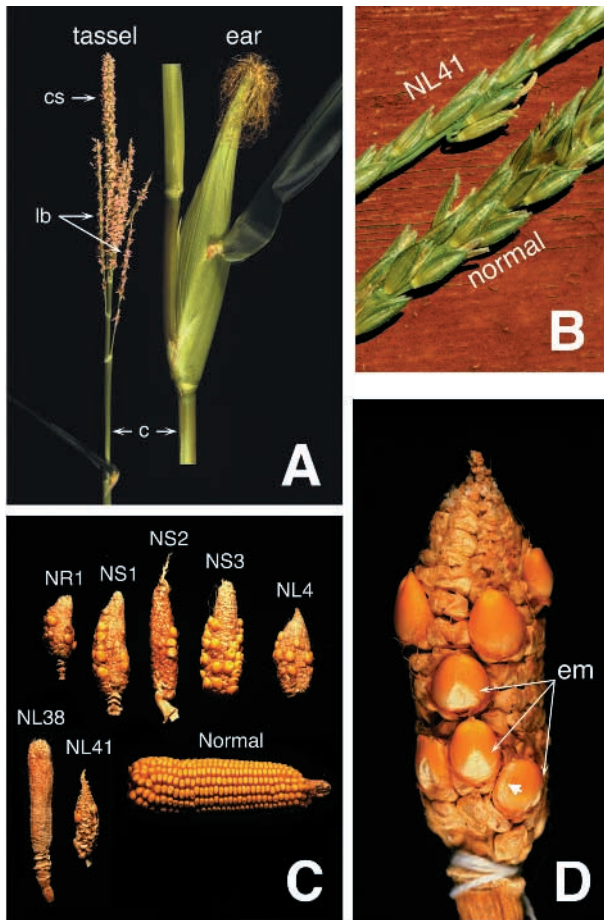


**Fig. 2.** Analysis of *kn1* protein and RNA expression. (A) The immunoblot was probed with a monoclonal anti-KN1 antibody. Each lane contains 350  $\mu$ g of soluble protein extracted from immature ears of plants homozygous for the indicated allele (except for *Kn1-N* which was heterozygous). KnN indicates the dominant progenitor, B73 the standard inbred line, and the recessive derivatives of *Kn1-N* (*kn1-NR1*, *kn1-NS1*, etc.) are indicated over their respective lanes. Molecular masses of prestained markers expressed as  $M_r \times 10^{-3}$  are indicated next to the first lane. An asterisk highlights the position of a faint band visible on the original blot. (B) The RNA gel blot was prepared from 10  $\mu$ g each of total RNA as described in Materials and Methods for the various alleles indicated as in A. The blot was hybridized with a probe from the 3' UTR of the maize *kn1* cDNA. (C) Rehybridizing the RNA gel blot with the oat  $\beta$ -tubulin cDNA allows the quantity of RNA in each lane to be compared.

maize seedlings homozygous for the various alleles. RNA gel blots, probed with the 3' untranslated region (UTR) of the *kn1* cDNA, revealed a single 1.7 kb transcript in RNA samples of all alleles shown except *NL41* (Fig. 2B). The *kn1* message is abundant in meristems of the dominant mutant progenitor *Kn1-N* and in the standard inbred line B73 (Fig. 2B). Greatly reduced levels of *kn1* transcript were detected in the loss-of-function mutants, though equal amounts of total RNA were loaded (see Fig. 2C). Nonsense mutations are frequently associated with reduced levels of mRNA. Some studies indicate premature translational termination can affect the rate of mRNA turnover (Losson and Lacroute, 1979; Urlaub et al., 1989; Peltz et al., 1993).

The *kn1* 3' UTR probe hybridized to novel sized RNA products in the *NL41* mutant consistent with a defect in splicing (Fig. 2B). The sizes of the two novel RNA species



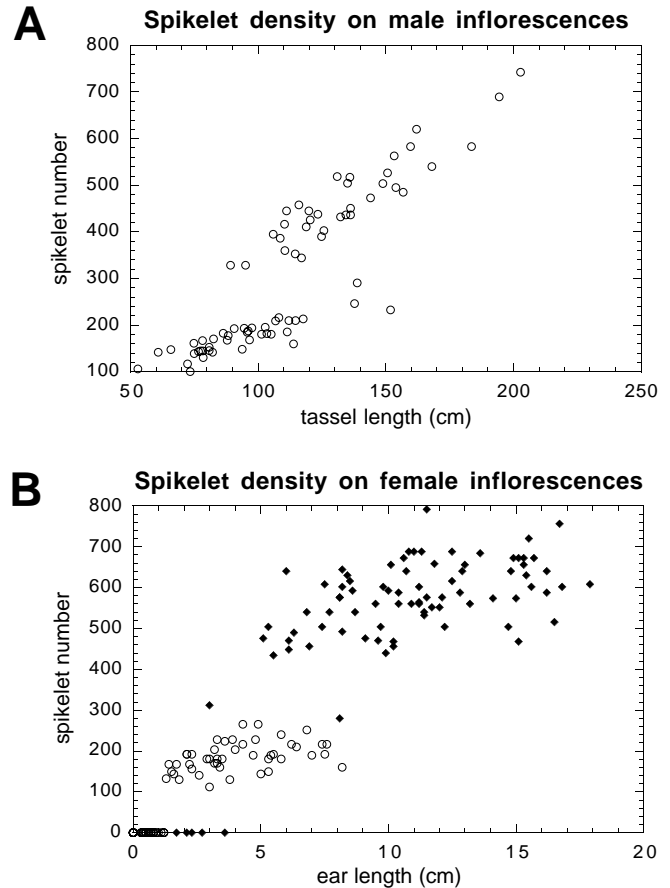


**Fig. 3.** Analysis of terminal and axillary inflorescence phenotypes of normal and *kn1* mutants. (A) Normal male and female inflorescences of the standard inbred line B73 are shown. The terminal inflorescence or tassel consists of a central spike (cs) and many long branches (lb) at the apex of the main axis or culm (c) of the plant. The primary axillary inflorescence or ear is enclosed in husk leaves with silks protruding. (B) A close-up view of a tassel branch from *NL41* and a normal sib are shown. Fewer pairs of spikelets for a given length of branch give the mutant tassel a sparse appearance. (C) Ears from some of the mutants and from a normal plant are shown. Differences between the mutant ears illustrate some of the variability in the phenotype rather than reproducible differences between alleles. (D) A close up of an ear from a self-pollinated *NRI* mutant reveals poor seed set and embryos (em) oriented in various directions on many of the kernels formed. The arrowhead indicates a white stripe on the kernel opposite the embryo.

detected are consistent with the predicted size of RNA molecules that would be produced if exon 2 was skipped entirely and if intron 1 failed to be spliced out. Surprisingly, the *NRI* mutant does not show novel mRNA species even though it carries the same base pair change as *NL41* at the next 3' intron splice junction downstream.

### Aspects of normal maize development

During maize development, a determinate number of vegetative leaves are initiated in a distichous phyllotaxis. At the floral transition, the vegetative SAM differentiates to form the staminate tassel (Fig. 3A) which terminates the shoot. At about



**Fig. 4.** Analysis of spikelet density on male and female inflorescences. The length of each inflorescence was plotted against the number of spikelets formed for both tassel (A) and ear (B) from a family segregating 1:1 for *NRI* heterozygotes and homozygotes. In A, the lengths of all long branches and the central spike (measured from the insertion point of the first long branch to the apex) were summed and all the spikelets were counted for 35 normal and 40 sparse tassels. In B, the length of each ear was measured from the basalmost spikelet to the apex, and the total number of spikelets was calculated as the product of the number of spikelets in one row by the number of rows. 84 plants with normal tassels (filled diamonds) and 96 plants with sparse tassels (open circles) were examined for ears. Fifty of the plants with sparse tassels had ears that failed to form or mature normally (represented by open circles that fall on the X axis or at the origin) compared to only 5 such ears found in plants with normal tassels. These 5 plants had normal ears at lower nodes whereas the 50 individuals with sparse tassels did not have any normal ear development.

the same time, one or more meristems in the axils of vegetative leaves begin to develop as pistillate lateral inflorescences, or ears, five to seven nodes below the tassel (Fig. 3A).

During the transition to flowering, the vegetative SAM elongates and initiates rows of branch primordia (Cheng et al., 1983; Stevens et al., 1986; Veit et al., 1993). Branch primordia at the base of the developing tassel form long primary branches which give rise to secondary branch primordia (lb in Fig. 3A). Branch primordia formed more apically on the main spike of the tassel and along the long branches (also known as spikelet pair primordia) give rise to a pair of short branches called

spikelets. Each spikelet branches yet again to form a pair of initially perfect grass flowers or florets. The ear develops in a similar manner: an axillary meristem first initiates a number of vegetative husk leaves and then elongates and initiates rows of spikelet pair primordia. Within each spikelet on the ear, the lower of the two florets undergoes programmed cell death (DeLong et al., 1993). The differential sexual character of maize flowers results from the late abortion of female reproductive structures (the pistil) in the tassel and the late arrest of male primordia (stamens) in the ear (Dellaporta and Calderon-Urrea, 1993).

### Recessive *kn1* mutants have reduced inflorescence branching

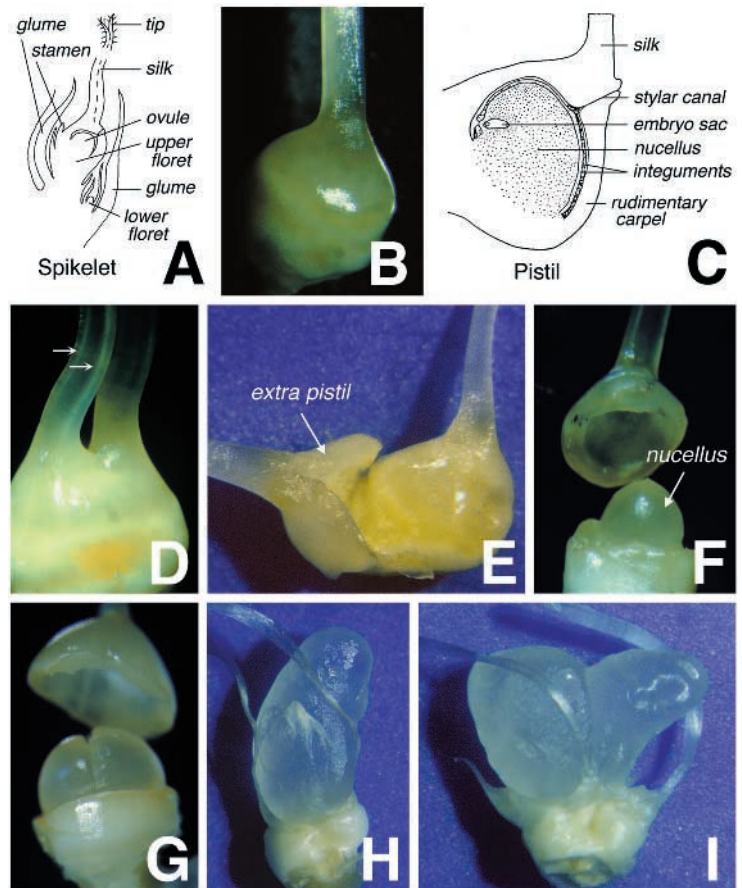
Plants homozygous for loss-of-function alleles of *kn1* display a variety of phenotypes in addition to the absence of knots on leaves. All of these alleles show the same spectrum of inflorescence and floral phenotypes. Ears are often absent or fail to develop on mutant plants and both the tassel and ears consistently show reduced branching (Fig. 3B,C). Fewer branch primordia form on male and female inflorescences which, in turn, results in significantly fewer spikelet pairs compared to normal sibs. Reduced inflorescence branching gives a sparse appearance to the mutant tassels (Fig. 3B), and the ears, when formed, appear smaller and shorter than normal (Fig. 3C,D).

Typical ears developing on homozygous mutant plants have at most 10 to 14 rows of flowers, most of which are sterile (Fig. 3D). The rows tend to be shorter as well, with only 25 to 40 flowers per row (Fig. 3D). In contrast, normal ears of the standard inbred line B73 have 40 to 50 fertile flowers in each of 14 to 18 rows. The small size of the mature mutant ear is due in part to sterility but also to the reduced number of spikelet pairs formed.

To quantitate the reduced branching in one of the *kn1* loss-of-function mutants, tassels were collected at anthesis from a family segregating for *NRI* heterozygotes and homozygotes. The lengths of the central spike and all long lateral branches of each tassel were measured, and the number of spikelets was counted. Fig. 4A shows two distinct clusters of tassels; the lower cluster represents the sparse tassels with reduced branching and the upper cluster represents normal tassels. Examination of many mutant tassels revealed that spikelets are paired as normal, though there are fewer pairs present (see Figs 3B, 4A) and the number of long branches is reduced. Homozygous plants have only about 60% of the primary tassel branches found in their heterozygous siblings. Examination of mature male flowers revealed no significant abnormalities compared to normal siblings (not shown).

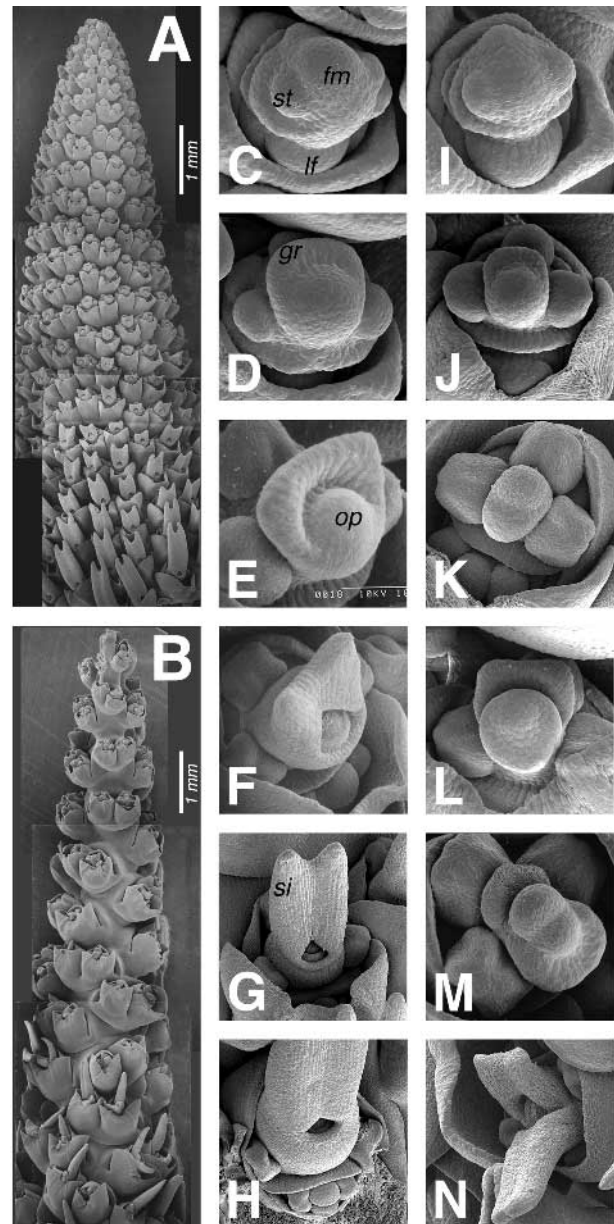
A similar analysis was done on the ears in the same family. Ear length was measured at anthesis and plotted against the number of spikelets. In Fig. 4B, two distinct clusters of data points are defined. The shorter overall length of the mutant ears may represent a combination of the reduced number of spikelet pair primordia initiated and a slight developmental delay, as the expansion of mutant ears lags behind normal sibling ears.

Ears frequently fail to develop normally in the axils of mutant homozygotes. Though not obvious in Fig. 4B, approximately half of the filled circles fall on or near the origin.



**Fig. 5.** Analysis of pistil and ovule phenotypes. (A) A median longitudinal section through a normal female spikelet (redrawn after Nickerson, 1954) is depicted. As in the tassel, the female spikelet consists of a pair of leaf-like glumes surrounding a pair of florets. Each floret consists of a lemma, palea, lodicules and three stamen primordia (which abort shortly after they are formed) and a pistil. The pistil terminates in an elongated silk. The lower floret aborts during the early stages of pistil initiation. (B) A normal pistil, consisting of a single ovary and silk, is shown. The glumes, lemma and palea have been removed from the florets shown in the following photographs. (C) A close-up of a radial longitudinal section through a normal pistil (after Randolph, 1936). Fusion of the styles of the two lateral carpels forms the silk which elongates rapidly. The margins of the lateral carpels and a third rudimentary carpel unite at the styler canal enclosing the ovule. (D) The pistil of an *NS2* mutant spikelet which produced an extra silk is shown. Four vascular traces (arrows) were present indicating extra carpels contributed to the formation of this structure. (E) Extra carpels form a partial extra pistil, containing no ovule, adjacent to an apparently normal pistil from an *NS3* mutant spikelet. (F) Incision through the carpel wall allowed the top of a normal pistil to be removed revealing the ovule inside. (G) A divided or double ovule from an *NRI* mutant is shown. The ovule was revealed by cutting through the carpel wall and removing the top of the pistil. (H) A pistil from an *NL4* mutant spikelet is shown. A mass of material resembling and continuous with nucellus tissue protrudes from between incompletely fused carpels. Two silks indicate extra carpels participated in the formation of this pistil. A thin, leaf-like structure, emerging from between the carpel and the proliferating ovule, formed in the position normally occupied by integuments. (I) An *NL4* mutant pistil with three silks has an ovule that is doubled and proliferating.

**Fig. 6.** SEM analysis of normal and mutant pistil development. A normal (A) and an *NL4* mutant (B) ear were each reconstructed from three micrographs. C to H show a series of stages in the development of normal pistils; the inflorescence axis is behind the flowers shown. (C) The floral meristem (*fm*) shortly after stamen primordia (*st*) have been initiated (*lf*, lower floret). (D) Initiation of a gynoecial ridge (*gr*). (E) The gynoecial ridge expands into a ring surrounding the apex (ovule primordium, *op*). (F) Two lateral carpels grow rapidly to form the silk and the ovule primordium initiates integuments. (G) The rudimentary and lateral carpels grow together to form the stylar canal and the elongating silk (*si*) becomes apparent. (H) A nearly mature pistil. The outer glume has been removed to show the lower floret with an aborted gynoecium. I to N show pistils in a variety of developmental stages from *NR1* and *NL4* mutants. Severe mutants all show the same floral defects. (I) An *NR1* floral meristem shortly after stamen primordia have been initiated. The floral meristems are slightly shallower than normal. (J) Initiation of a gynoecial ridge from an *NR1* meristem. The gynoecial ridge appears later (as judged by the shape of anther primordia that continues to progress) and is slightly narrower than normal. (K) A very delayed (possibly arrested) pistil primordium (*NL4*). Anther primordia appear very mature. (L) After initiating a gynoecial ridge, the *NR1* floral meristem initiates a second gynoecial ridge not quite opposite the first. (M) After initiating two gynoecial ridges, the floral meristem (*NL4*) has formed another primordium (possibly a second ovule primordium). (N) Silks elongate from both of the gynoecial ridges that were initiated in this *NL4* floret.



These represent homozygous mutant plants that were either missing an ear or had small ear primordia between 0.5 and 1.5 cm in length (with small spikelet primordia that were not counted).

#### Extra carpels and proliferating ovule tissue form in mutant female flowers

Mutant flowers were examined at the time of pollination and numerous defects were discovered. Not all flowers show obvious defects, which may account for the partial fertility on mutant ears. The normal maize pistil (Fig. 5A,B) is thought to consist of three carpels fused edge to edge (Walker, 1906; Randolph, 1936; Irish and Nelson, 1993). Vascular bundles of the two lateral carpels pass into the silk while the third carpel normally remains rudimentary (Fig. 5C) with but a vestigial vascular trace (Walker, 1906). Female flowers on mutant ears may produce no pistil at all, an apparently normal pistil, or a pistil with varying numbers of extra carpels (Fig. 5D,E). The initiation of extra carpels is usually reflected in extra silk production. Extra silks in the mutant flowers often have two vascular traces and a bifurcated tip suggesting the extra silk is formed from at least two extra carpels (Fig. 5D). In some cases, more than one extra silk is made or additional pistil-like structures form. These ancillary pistils are usually incomplete and lack ovules (Fig. 5E). A single female inflorescence may show all of these types of flowers or only a subset, and the number of each type of defective flower varies from ear to ear (not shown).

In addition to extra carpels, pistils in mutant flowers may have unusual ovules. The normal maize ovule consists of maternal nucellus tissue that is enclosed entirely by inner and partially by outer integuments and completely fills the locule formed by the fused carpels (Fig. 5C,F). In mutant florets, pistils can contain no ovule, an apparently normal ovule, an

extra or divided ovule, a proliferating ovule, or both extra and proliferating ovules (Fig. 5G,H,I).

In order to discover when mutant female flowers first deviate from normal development, immature ears were examined by scanning electron microscopy (SEM). During normal floral development (Fig. 6A), shortly after stamen primordia have been initiated in the upper floret (Fig. 6C), a ridge of tissue arises on the side of the floral meristem nearest the inflorescence axis (Fig. 6D). This gynoecial ridge represents the beginning of a ring of carpel primordia fused from inception. Carpel primordia extend to encircle the floral meristem which, at this stage, can be considered an ovule primordium (Fig. 6E-F). The two lateral carpels undergo more rapid growth and form the silk (Fig. 6F-G). The ring of carpel tissue ultimately overgrows and encloses the ovule (Fig. 6F-H). The point where lateral carpels and the top of the rudimentary third carpel meet forms the stylar canal (Fig. 6H).

SEM on mutant ear primordia revealed that the number of rows of spikelet pairs is markedly reduced and the space between successive spikelet pairs is increased in *NL4* mutant ears (Fig. 6B). Spikelet and floral meristem development follow the normal pattern of events through the initiation of stamen primordia, after which, the floral meristem deviates from normal development. After the initiation of stamen primordia, the mutant floral meristem is initially flattened compared to normal (Fig. 6I) and formation of a gynoecial ridge is delayed (Fig. 6J). A narrow gynoecial ridge eventually does form in mutant flowers (Fig. 6J). A small percentage of flowers fail to proceed beyond this point (Fig. 6K) while others follow a relatively normal developmental path (not shown). In many cases, the floral meristem continues to enlarge and initiates a second gynoecial ridge nearly opposite from the first (Fig. 6L). The floral meristem may continue to initiate structures, either forming more carpel primordia or one or more ovule primordia (Fig. 6M). The extra carpels fuse partly or entirely with the first carpels formed, often resulting in a single pistil with two silks (Fig. 6N) and occasionally resulting in more than two silks or even extra pistils (see Fig. 5).

### Mutant plants make extra vegetative leaves

A vegetative phenotype has also been observed in some plants homozygous for loss-of-function mutations. An extra portion of leaf is found in the axil of an otherwise normal leaf at one or more nodes (Fig. 7). While this phenotype is only partially penetrant, it occurs in mutant plants grown under many different conditions and not in normal plants (see below).

During normal vegetative development, leaves initiate in a distichous phyllotaxis with one leaf per node. Each leaf consists of sheath at the base, which wraps around the stem, and blade (Fig. 7A). In mutant plants, an extra portion of leaf is found inserted at the same node as the normal leaf which immediately subtends and surrounds the extra leaf (Fig. 7B,C,D). Though the extra leaf portion is always narrower than a normal leaf, it may extend the full length of the normal leaf or be substantially shorter, in some cases, consisting entirely of sheath (Fig. 7D). The extra portion of leaf may be fused with the normal leaf beneath it for some or all of its length (Fig. 7C), or it may be completely free (Fig. 7B). When the extra leaf is fused to the normal leaf, ligule and auricle, which mark the boundary between sheath and blade, intersect at the point of fusion. When unfused, the auricle forms a broad band across the extra leaf rather than converging to a point at the midvein of the leaf (Fig. 7B).

The extra portions of leaf lack a defined midvein and are typically inserted slightly off center from the midvein of the subtending leaf. The extra leaf occurs most often between the ear node and the top leaf in mutant plants, appearing occasionally on the leaf subtending an ear (not shown). Examination of surface characters of the extra leaves show that they are reversed relative to the subtending leaf, such that the adaxial leaf surfaces of the normal leaf and extra leaf face each other (not shown).

Not every plant homozygous for a *kn1* loss-of-function allele shows an extra leaf. Plants that do make an extra leaf have one or more affected nodes, and fused, unfused, and sheath-only types of extra leaf may occur at different nodes of the same plant. We examined three large families (total 540 plants) for extra leaves in order to quantitate the penetrance of this

phenotype. Eight - 12% of the mutants showed extra leaves, while normal plants did not show the extra leaf phenotype.

## DISCUSSION

Analysis of knotted leaves in maize and the phenotypes of transgenic tobacco and *Arabidopsis* plants expressing high levels of the maize cDNA have pointed toward roles for *kn1* in cell fate acquisition and the switch between determinate and indeterminate fate in the SAM (Smith et al., 1992; Sinha et al., 1993; Chuck et al., 1996). To further elucidate the role of *kn1* in development, recessive alleles at the maize *kn1* locus were generated by mutagenesis of a dominant mutant allele and subsequently characterized. Plants with the recessive *kn1* alleles no longer show the knotted leaf phenotype and instead display a number of phenotypes present neither in normal maize plants nor in the dominant mutant progenitor. The loss-of-function alleles at *kn1* result in three distinct phenotypes: reduced inflorescence branching, proliferating pistils, and extra portions of vegetative leaves. Analysis of these phenotypes indicates KN1 has a significant role in meristem maintenance in the developing maize plant.

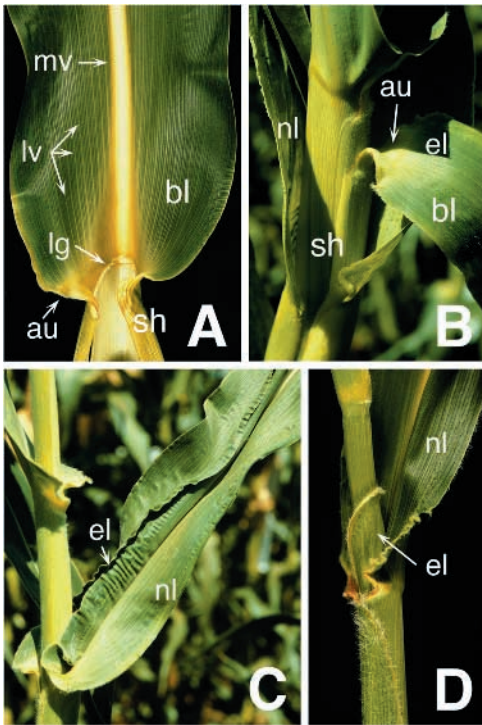
### Different base pair changes disrupt KN1 function

Four of the recessive alleles of *kn1* generated in this study affect RNA splicing; *NL41* and *NR1* have defects in splice acceptors while *ND35* and *ND48* affect splice donors. The *NL41* and *NR1* alleles both carry G to A transitions in the AG dinucleotides of different intron splice acceptors. In the *NL41* mutant, a 3' UTR probe detected RNA products (Fig. 2B, lane *NL41*) consistent with the failure to splice intron 1 and the skipping of exon 2. Surprisingly, RNA products resulting from the failure to splice intron 2 or the skipping of exon 3 were not detected in the *NR1* mutant. Detection of reduced levels of apparently normal size *kn1* mRNA and protein in the *NR1* mutant (Fig. 2A,B) may indicate that a cryptic splice acceptor is utilized, producing an in-frame deletion or insertion of one or more amino acid residues. Since *NR1* homozygous plants have a severe loss-of-function phenotype, this result may indicate that the specific residues or spacing of protein domains in the vicinity of the 5' end of exon 3 are critical for *kn1* function.

The frame shift and nonsense alleles (Fig. 1) are predicted to result in truncated KN1 polypeptides. The frame shift resulting from the 2 base pair insertion found in the *D40* allele is predicted to produce a short peptide of 48 amino acids of which only the first 15 share identity with KN1. The various nonsense alleles (*NS1*, *NS2*, *NS3*, *ND6*, *NL4*, and *NL38*) are predicted to form proteins smaller than KN1, some of which were detected on immunoblots with a monoclonal anti-KN1 antibody (Fig. 2A). Since different nonsense mutations at various locations in the coding sequence give essentially the same mutant phenotype, these alleles may reasonably be considered null alleles. Preliminary characterization of the *NL29* mutant suggests it may be an exception. Plants homozygous for *NL29* appear to have a less severe phenotype (data not shown). Experiments to further characterize the phenotype of *NL29* plants are in progress.

Among the nonsense mutants, *NL4* and *NL38* are predicted to form proteins 76% and 49% of the normal size of KN1





**Fig. 7.** Loss-of-function mutants have an extra leaf phenotype. Various types of ‘extra leaf’ form in a small percentage of the plants homozygous for severe alleles. A normal leaf removed from the culm is shown in A. The sheath (sh), blade (bl), midvein (mv), lateral veins (lv), ligule (lg), and auricle (au) are indicated. B shows an unfused extra leaf blade (el) protruding from between the culm and a normal leaf (nl) of the *NS3* mutant. In C, the extra portion of leaf (el) is fused to the normal leaf (nl) subtending it, off center from the midvein, in an *NL38* plant. D shows an extra portion of leaf (el) from an *NRI* plant that consists only of sheath tissue with no blade. In some cases, all three types of extra leaf appear on an individual plant (not shown).

respectively. Proteins consistent with these sizes were detected in immunoblots while alleles predicted to truncate KN1 further upstream were not detected. It is likely that smaller peptides are either less stable or they lack specific epitopes for detection with the monoclonal antibody. Much more of the shorter KN1-NL38 protein was detected in the immunoblots than KN1-NL4 (Fig. 2A). The presence of a putative PEST sequence in NL4, not present in NL38, may account for the apparent difference in accumulation of the two peptides. PEST sequences are found in a number of proteins that form multimeric complexes (Rogers et al., 1986). Dissociation of multimeric proteins is thought to lead to rapid proteolysis of the subunits when PEST sequences that are normally covered are exposed (Rogers et al., 1986). The presence of the PEST region in KN1 may indicate that the protein is rapidly degraded in the absence of a partner with which it interacts. The recent identification of a potential protein-protein interaction module in KN1 (Mushegian and Koonin, 1996) is also suggestive of an interaction between KN1 and other proteins.

### Role of KN1 in inflorescence branching

The partial loss of inflorescence branches is one of the more striking features of the *kn1* loss-of-function mutant plants. The

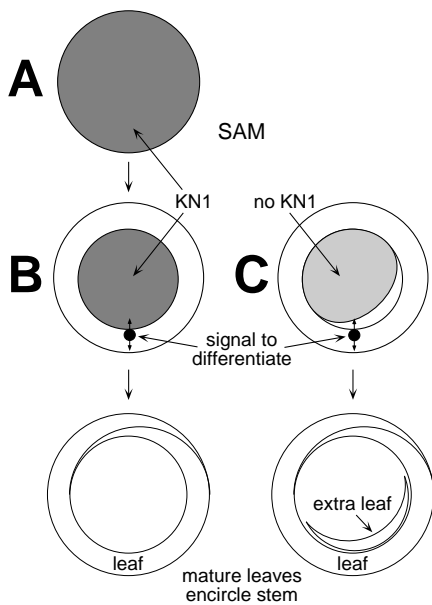
number of long branches and the number of spikelet pairs are reduced in the tassel (Fig. 4A), and spikelet pairs are similarly reduced in the ear (Fig. 4B). This loss results in somewhat smaller inflorescences with fewer flowers (Fig. 3C-F). The defect is specific to branches forming from the inflorescence meristem. We propose a model for how loss of KN1 in the inflorescence meristem might affect the formation of branch primordia by reducing the capacity of the meristem to maintain itself. If KN1 is required to help maintain cells in a meristematic or undifferentiated state, loss of KN1 activity may lead to an ever shrinking pool of meristem cells in the inflorescence apex. Fewer inflorescence meristem cells would be available to contribute to the initiation of branch and spikelet pair primordia. In fact, long branches which form at the base of the male inflorescence appear to be less severely affected than spikelet pairs, as if the loss of branch initiation capacity is progressive. Tassels of the *NR1* mutant produce about two-thirds the number of long branches as heterozygous sibs but only one-third the number of spikelet pairs.

An alternative model may be proposed wherein KN1 plays a role in establishing meristematic identity in initiated branch primordia. Expression studies during normal development indicate KN1 is expressed throughout the dome of the inflorescence meristem and is down-regulated on the flanks of the inflorescence meristem in a regular pattern that appears to predict the position of the next branch primordium (Smith et al., 1992; Jackson et al., 1994). Shortly thereafter, just as the branch primordia become visible, KN1 expression appears in the dome of the branch meristem. One interpretation of this expression pattern is that KN1 is down-regulated in the inflorescence meristem to initiate branch primordia, just as occurs during initiation of lateral organ primordia, but KN1 expression must be subsequently re-established in that primordium if it is to adopt and maintain a meristematic fate. In the absence of KN1 activity, re-establishing meristem identity in branch primordia may be less regular, resulting in fewer lateral branches. The absence of ear primordia from many of the mutant plants may result from a similar process if the meristematic identity of axillary buds initiated during vegetative development fails to be established or properly maintained. Since many ears and inflorescence branches do form in the mutants, a partially redundant factor may sometimes compensate for the absence of *kn1*.

### KN1 has a role in floral determinacy

Pistil formation is significantly perturbed in the *kn1* loss-of-function mutants. In the absence of KN1, extra whorls of carpels are frequently initiated resulting in a pistil-in-pistil arrangement or fusions to form large pistils with extra silks or ancillary pistil-like structures. The defect specifically affects the determinacy of the carpel and ovule, since extra floral organs were not detected in mature male spikelets where the pistil aborts, nor were extra stamen primordia observed in immature ears examined by SEM (data not shown). The loss of determinacy in the female floral meristem is analogous to that observed in the *agamous* mutant of *Arabidopsis* (Yanofsky et al., 1990), which has been shown to affect floral meristem determinacy, in addition to (and separable from) its effects on stamen and carpel organ identity (Mizukami and Ma, 1995; Sieburth et al., 1995). In fact, a mutation in the maize homologue of *AGAMOUS*, *zag1*, produces ears with two silks





**Fig. 8.** Model for the activity of KN1 in maintaining the boundary between differentiating and undetermined cells in the meristem. (A) KN1 marks all of the cells in the meristem (indicated by shading). (B) KN1 is down-regulated in cells that will contribute to the next lateral organ. These cells are now competent to respond to signals to differentiate and form a leaf primordium. Cells in the meristem that continue to express KN1 are resistant to the signals to differentiate. (C) In *kn1* mutants, the absence of KN1 makes cells in the meristem vulnerable to signals to differentiate, leading to the formation of an extra portion of leaf. (Lighter shading indicates a redundant factor providing weak KN1 activity.)

developing in the pistil (Mena et al., 1996). Although the *zag1-mum1* extra silks phenotype bears a striking similarity to that observed in *kn1* mutant florets, the reduced inflorescence branching and the vegetative extra leaf phenotype in *kn1* mutants were not present in the *zag1* mutants (our unpublished observations; Mena et al., 1996) and extra or divided ovules are also unique to the *kn1* mutant phenotype. Determining whether *kn1* and *zag1* interact specifically in pistil formation should prove interesting. A precedent for the interaction of a homeodomain protein and a MADS protein exists in the transcriptional regulators MAT $\alpha$ 2 and MCM1 of yeast (Herskowitz, 1989).

### KN1 and meristem boundaries

The extra leaf phenotype, though poorly penetrant, may provide some of the strongest clues into the normal role of KN1. The production of extra organs as an outcome of the loss of meristem specific expression resembles in some ways the normal events that occur as leaves are initiated. Fig. 8 illustrates a model for how KN1 could mark a boundary in the SAM between cells that will remain meristematic and cells that will respond to signals to differentiate into lateral organs. In a normal meristem (A), KN1 is down-regulated in a localized portion of the SAM, corresponding to where the next leaf primordium will initiate. Cells no longer expressing KN1 become competent to respond to differentiation signals, while KN1 prevents cells in the rest of the meristem from responding (B). In the loss-of-function mutants, the boundary between cells

competent to respond to differentiation signals and resistant cells may no longer be strictly maintained (C). Cells in the meristem that would otherwise resist the signals to differentiate and remain meristematic may be induced to form organ primordia in the absence of KN1, resulting in extra organs. This model could account for the initiation of extra vegetative leaves and the extra pistils observed in female florets. Since the SAM is not completely consumed by organ initiation, other gene products may function in a manner similar to KN1 to prevent cells in the SAM from responding to signals to differentiate.

A related gene in *Arabidopsis*, *SHOOT MERISTEMLESS* (*STM*) has a pattern of expression very similar to *kn1* (Long et al., 1996). *STM* expression is first detected in a few cells in the center of the apical half of early globular embryos (Long et al., 1996). Severe alleles result in the absence of a histologically defined embryonic SAM, though other embryonic structures form including cotyledons, hypocotyl and root (Barton and Poethig, 1993). The phenotypes of weak alleles of *STM* have also been characterized (Clark et al., 1996; Endrizzi et al., 1996) and they share some similarities with the *kn1* loss-of-function mutants including reduced numbers of flowers. The weakest mutant characterized, *stm-6*, shows an excess of carpels in about half the flowers, with many of the carpels incompletely fused (Endrizzi et al., 1996). This resemblance may indicate that the loss of *kn1* uncovers a redundant factor in the maize genome with weak activity, and leads us to predict that double mutants in *kn1* and the redundant factor may result in a phenotype similar to that of severe *stm* mutants. *kn1* is one member of a family of closely related *knox* genes in maize, many of which show patterns of expression suggestive of related roles in maize meristems (Jackson et al., 1994; Kerstetter et al., 1994). The normal role of *kn1* may be broader than that revealed by loss-of-function mutants if expression of other *knox* genes can partially compensate for its loss.

In conclusion, we propose that the maize *kn1* gene product serves to maintain cells in a meristematic or undifferentiated state. Cells expressing KN1 display some resistance to differentiation signals and serve as a population from which cells are recruited for organ primordia formation. KN1 may also be involved in the establishment of meristematic identity in branch primordia. These proposed functions of KN1 appear to be at least partially redundant in maize and are consistent with the dominant mutant and transgenic phenotypes.

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