Development 122, 1683-1691 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
DEV0067

The maize mutant *narrow sheath* fails to establish leaf margin identity in a meristematic domain

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SUMMARY

The maize mutant *narrow sheath* (*ns*) displays a leaf shape and plant stature phenotype that suggests the preprimordial deletion of a leaf domain. The *ns* mutant phenotype is inherited as a recessive, duplicate-factor trait, conditioned upon homozygosity for each of the two unlinked mutations *narrow sheath-1* (*ns1*) and *narrow sheath*-2 (*ns2*). Mutant leaves are missing a large domain including the leaf margin, and mutant internodes are shortened on the marginal side of the stem. This domain deletion extends from the internode to beyond the longitudinal mid-length of the blade, and corresponds to an alteration in the organization of a specific region of the shoot apical meristem. The premargin region of mutant founder cells fail to down-regulate expression of *Knox* genes, markers of nonleaf meristematic identity. Our results indicate that leaf domains may acquire identity in the meristem itself, and that the subdivision of preprimordial developmental fields into differential domains is a common feature of both plant and animal organogenesis.

Key words: *narrow sheath*, maize, domain, meristem, leaf margin, preprimordial deletion

INTRODUCTION

The maize leaf is a photosynthetic organ comprising three basic parts; the proximal sheath which arises from the node and surrounds the culm (stem), the distal blade which projects outward from the culm, and the ligular-auricular region which serves as a hinge between the blade and sheath. The leaf is one component of a phytomer, the basic, repeating structural unit of the maize plant (Galinat, 1959). A maize phytomer comprises the basal internode and lateral bud (the tiller, or the ear with its prophyll), the node, and the leaf. In maize, the tubular base of the leaf extends into the node and internode, both of which are derived from the 'disc of insertion' of the primordial leaf (Sharman, 1942; Kaplan, 1973).

Successive maize leaves are initiated on the flanks of the vegetative shoot apical meristem in a distichous pattern (180 degrees apart and in two ranks). The unit of time between initiation of successive leaves from the meristem is termed a plastochron (Sharman, 1942). A leaf primordium is described as a plastocron 2 [P2] leaf when it is the second leaf from the shoot apex. The next youngest leaf primordium is termed a [P1], and the preprimordial founder cell population within the meristem is, therefore, termed a [P0]. Lineage mapping studies utilizing genetic mosaics have demonstrated that the maize phytomer initiates from approximately 200 founder cells that form an overlapping ring within the shoot apical meristem (Poethig, 1984). The accumulation patterns of the KNOTTED1 (KN1) maize homeodomain protein and related proteins such as ROUGH SHEATH1 (RS1) provide useful markers for leaf identity in the shoot apical meristem (Smith et al., 1992; Jackson et al., 1994; Schneeberger et al., 1995). KN1 accumulates to high levels in the corpus of the meristem but is down regulated in leaves and leaf primordia. Down regulation of KN1 occurs first in a ring of cells surrounding the shoot apical meristem whose position and histology identify them as founder cells of the incipient leaf (Smith et al., 1992, 1995; Jackson et al., 1994). Similar patterns of expression are reported for *stm1*, the putative *kn1* orthologue in *Arabidopsis* (Long et al., 1996).

Following founder cell initialization the primordial stage of leaf development ensues, whereupon all founder cell derivatives divide approximately equally to form a 4 mm long [P3] primordium (Poethig, 1984). This unanimous and equal division in the epidermis of leaf primordial cells was later confirmed by Sylvester et al. (1990) using scanning electron microscopy (SEM) to measure the depths of cellular crosswalls and quantify cell division indices. Sharman (1942) demonstrated that the complete complement of lateral veins are present at the end of the primordial stage of leaf growth. Post-primordial growth of the maize leaf is characterized by an early expansive growth of the blade, followed by an elaboration of the basal sheath (Sylvester, 1990; Sharman, 1942). Thus, the maize leaf differentiates basipetally.

Several aspects of plant development are fundamentally different than that of animals. These include the absence of somatic cell rotation or migration, non-sequestration of the germ line, the maintenance of undifferentiated cells (meristems) in post-embryonic stages, and cell fates that are

usually dependent upon cell position, rather than cell lineage (reviewed by Steeves and Sussex, 1989; Dawe and Freeling, 1992; van den Berg et al., 1995)). A keynote feature of animal development is the subdivision of organ primordia into developmental compartments (Garcia-Bellido et al., 1973). First identified by their boundaries of cell-lineage restriction, compartments were later shown to arise via the expression of transcriptional activators in particular regions (e.g. Kornberg et al., 1985). The boundaries of neighboring compartments serve as organizing centers for pattern formation in the developing organ (Basler and Struhl, 1994; Williams et al., 1994; Tabata et al., 1995). The applicability of this animal strategy of piecemeal organogenesis has not been tested in plants.

Analyses of several dominant mutants altering maize leaf regional identities indicate that this organ may be a patchwork of semi-independent 'domains' (a term we use to denote leaf regions identified by the expression of various mutant phenotypes) (Freeling, 1992). A model for maize leaf development has been described whereby discrete domains within the meristematic leaf founder cells are initialized to assume regional identities (Freeling, 1992). If the maize leaf is indeed a patchwork of domains, then one might expect that the phenotypes of particular, recessive pattern mutants would remove a patch by disrupting the initialization of meristematic cells. Here we describe such a domain deletion mutant, *narrow sheath (ns)*. Inherited as a duplicate factor recessive trait (*ns1*;*ns2*) *ns* mutant plants exhibit the loss of a leaf margin domain that is correlated with an altered pattern of homeobox gene expression in a region of the meristem. We propose that the role of the *ns* gene product is to initialize leaf founder cells in a specific, meristematic domain. Our results suggest that a commonality of developmental design is shared among the plant and animal kingdoms. That is, organogenesis in both groups proceeds via subdivision of an existing developmental field into differential domains, followed by elaboration of these preprimordial patterns in a semi-independent but coordinated fashion.

MATERIALS AND METHODS

Maize stocks and genetic analyses

The *ns* mutant phenotype was identified by E. Elsing and M. Albertson of Pioneer Hi-Bred Intl., Johnston, IA. The line in which the *ns* phenotype was first identified is denoted as the *ns* 1:1 line, and was a gift from Pioneer Hi-bred Intl. Nonmutant stocks used as standard lines included the inbreds B73 (Pioneer Hi-bred Intl.), Mo17, W23, and Q66 (obtained from D. S. Robertson, Iowa State University).

The mode of inheritance of the *ns* phenotype was determined by crossing *ns* mutant plants to various maize inbreds, self-pollinating the F_1 progeny, and screening for the presence of the *ns* mutant phenotype in the F_2 progeny. In most cases the F_2 progeny were screened as seedlings because the *ns* mutant phenotype is fully penetrant in 1-week-old seedlings.

Microscopy

Free-hand sections of adult leaf margins were examined under UV microscopy as described previously by Becraft and Freeling (1994). Scanning electron microscopy (SEM) was performed on replicas of 2-week-old *ns* mutant and nonmutant sibling plants obtained from the *ns* 1:1 line (see Materials and Methods, Maize Stocks) as described by Sylvester et al. (1990). Samples were coated with 25 nm of gold using a Polaron E5400 high-resolution sputter coater and analyzed on a ISI DS-130 SEM with a LaB6 filament operating at 15 kV. Estimates of the division indices, given as the fraction of cells that have undergone recent cell division, for [P2] and [P3] leaf primordial cells were performed as described by Sylvester et al. (1990).

Preparation of KNOX antibody

Polyclonal antisera that recognize KNOX (Kerstetter et al., 1994) proteins were raised against a bacterially expressed protein representing the carboxy-terminal 152 amino acids encoded by the *rough sheath1* gene beginning 55 amino acids proximal to the homeodomain. This region contains the 63 amino acid homeodomain which shows 85% amino acid identity to the KNOTTED1 amino acid sequence (Schneeberger et al. 1995). The ROUGH SHEATH1 polypeptide was expressed as a glutathione S-transferase (GST) fusion protein in BL21 cells (using methods detailed by Smith and Johnson, 1988). Bacterial cultures carrying a pGEX2T-RS1 fusion construct were induced with IPTG and GST-RS1 fusion proteins and purified from lysates on a glutathione sepharose column (Pharmacia). The RS1 polypeptide was purified from GST by thrombin protease cleavage (SIGMA) and subsequent elution from the glutathione-sepharose column (Smith and Johnson, 1988; Pharmacia). The purified RS1 polypeptide was used to raise antisera in New Zealand white rabbits according to standard protocols (Harlow and Lane 1988). RS1 polyclonal antisera was purified on an agarose, carbonyldiimidazole cross linked, RS1 protein affinity column (Pierce; Hearn et al., 1981). Western blots of bacterially expressed RS1 and KNOTTED1 (KN1) protein (Smith et al., 1992) were incubated with anti-RS1 sera (RS506) and showed high cross reaction to KNOTTED1 (Fig. 1A, lanes 1 and 2). Antibodies that recognize common epitopes in both the RS1 and KN1 proteins were then isolated by affinity chromatography on a KN1 affinity column and are termed KNOX antibodies (Fig. 1, lanes 5 and 6). This chromatography step also produced antibodies that only recognized RS1 (RS506-KN; Fig. 1A, lanes 3 and 4). Immunodetection of immature ear protein extracts with the KNOX and RS506-KN antibody shows that two protein species are identified by the KNOX antisera which correspond to RS1 and KN1 (Fig. 1B; compare lanes 1 and 2). Immunolocalizations of the KNOX antibody preparation show a pattern indistinguishable from that of KNOTTED1 in the leaf initiation regions of the shoot apical meristem (Smith et al., 1992) (Fig. 5 and R. Schneeberger, unpublished data). Maize protein isolation, SDS-PAGE electrophoresis and western blotting were performed as described by Donlin et al. (1995).

Immunohistology

Immunohistochemistry was performed as described by Smith and coworkers (1992), and immunopositive nuclei were visualized using colorimetric methods as described by Jackson et al. (1994). Where indicated samples were counterstained with Basic Fuchsin.

RESULTS

Morphology of *ns* mutant plants

The *ns* mutant is a brachytic (short internode) plant with extremely narrow leaves. Mutant leaves are narrow immediately above the region of leaf insertion, throughout the sheath, and much of the blade (Fig. 2B,C,E). Whereas the margins of nonmutant leaf sheaths overlap immediately above the node, *ns* mutant sheaths expose the stem (Fig. 2F,G). Beyond the mid-length of the blade *ns* leaves gradually widen but never attain nonmutant leaf width (Fig. 2B). Despite the reduction in leaf width, the length of *ns* mutant leaves is similar to nonmutant siblings (Fig. 2B). The husk leaves of the female

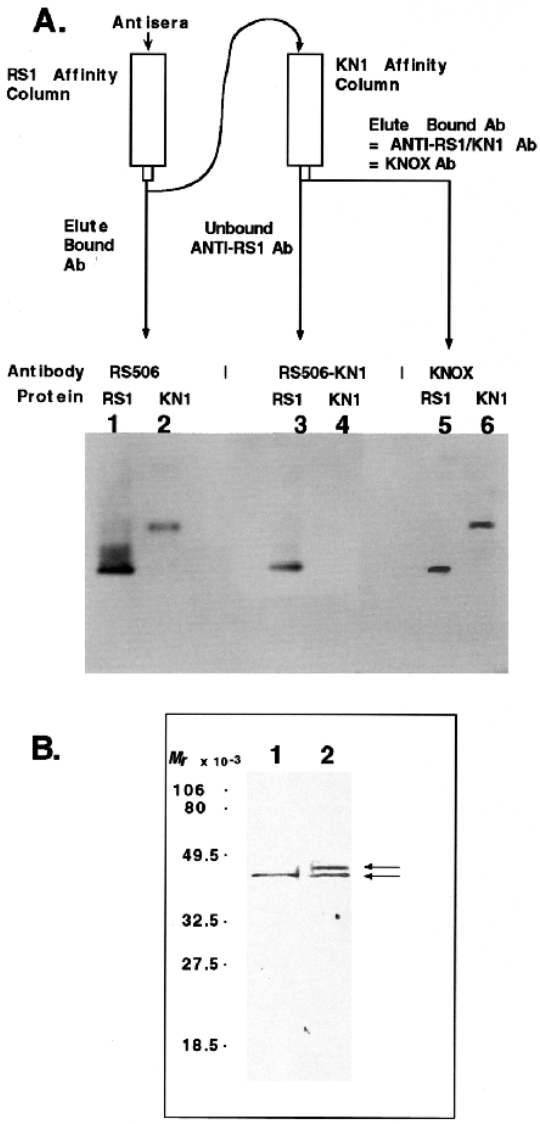

Fig. 1. KNOX antibody preparation. (A) Purification scheme used to isolate RS1 (RS506-KN) and KN1 and RS1 common epitope antibodies (KNOX) and SDS-PAGE analysis of their activities. Immune serum directed against the RS1 protein (see Materials and Methods) was fractionated by sequential passage over two affinity columns. Fractions from each step of purification, represented by the arrows, were used to probe western blots containing 1 ng of bacterially expressed RS1 and KN1 proteins (Smith et al., 1992). RS1 affinity purified antiserum (RS506) detects both RS1 and KN1 proteins (lanes 1 and 2). Removal of epitopes common to both KN1 and RS1 produces an antiserum that recognizes only RS1 (RS506-KN, lanes 3 and 4). The antibodies eluted from the KN1 affinity column (KNOX) recognize both KN1 and RS1 proteins in approximately equal intensity (lanes 5 and 6). (B) Immunodetection of RS1 and KN1 proteins in immature ear protein extracts. Lanes 1 and 2; 10 μg of immature ear protein were electrophoresed on a 4-15% gradient SDS-PAGE gel and blotted onto PVDF membrane. Lanes 1 and 2 were incubated separately with RS506-KN (lane 1) and KNOX (lane 2) antiserum respectively and detected using alkaline phosphatase-conjugated anti-rabbit IgG secondary antibody (Biorad). Note that the KNOX antiserum detects two bands and RS1 antiserum detects one band. The upper band represents the KN1 protein as determined by stripping and reprobing lane 2 with a KN1 specific antiserum (data not shown).

inflorescence (ear) and the leaf homologues of the male flower (glumes, lemmas and paleas) are similarly affected in *ns* mutants (data not shown). Moreover, the number of lateral veins in *ns* mutant leaves is usually one-half to one-third of that found in nonmutant sibling leaves (Fig. 2C). This reduction in leaf width is not achieved via compression; the number of intermediate veins between lateral veins, intervein spacing and Kranz anatomy of mutant leaves is unaffected.

The narrow leaf phenotype exhibited in *ns* mutant plants is most severe in more basal leaves (L1-L13), and thereafter (L14-L20) is increasingly alleviated in successive leaves approaching the tip of the plant (Fig. 2E). The alleviation of the leaf phenotype is first manifested as a gradual decrease in the degree of leaf blade deletion, followed by a similar phenomenon in the sheath. Although the leaves of *ns* plants widen in upper nodes, they never attain wild-type width. The *ns* mutant flagleaf (usually L20, defined as the uppermost leaf immediately below the tassel) is slightly narrower than that of nonmutant siblings. A curious *ns* phenotype frequently found in upper phytomers is asymmetry about the midrib (data not shown). Manifested as a difference in leaf width to the right versus the left of the midrib axis, the asymmetry phenotype is also restricted to the proximal half of affected leaves.

Two additional features of the *ns* mutant phenotype are stem curvature (Fig. 2H,I) and shortened internodes (Fig. 2F,H,I). In the curved, mutant stems, the side of the internode corresponding to the margin of the leaf it subtends is shorter than the opposite side of the internode, which corresponds to the leaf midrib. This nonuniform internode growth pattern results in serpentine stems (Fig. 2H). Although the longitudinal expansion of the internode is affected by the *ns* mutations, there is no detectable effect on internode lateral growth. That is, the marginal side of the *ns* internode is not abnormally flattened as compared to nonmutant siblings. Although the internode shortening phenotype is alleviated in upper phytomers of *ns* plants, some degree of curvature persists in all mutant stems (Fig. 2I).

Anatomical evidence indicates *ns* mutants lack margin structures throughout the affected leaf regions. These include the distinctive sawtooth hairs that develop at the margins of nonmutant leaves beginning at the blade/auricle junction (Fig. 3G,H), which are absent in *ns* mutants until beyond the mid-length of the blade. Moreover, the edges of wild-type leaves contain a distinctive structure at the margin (not simply an abutment of abaxial (lower) and adaxial (upper) surfaces) characterized by a chlorophyll-free, tapered expanse which is longest in the sheath (Fig. 3E), and shorter in the proximal half of the blade (Fig. 3C). In contrast, *ns* mutant leaf sheaths (Fig. 3F) and proximal blades (Fig. 3D) form blunted edges. Intriguingly, the proximal blade of *ns* mutants forms an edge by an apparent folding of the abaxial surface onto the adaxial surface, as evidenced by the curved arrangement of the veins at the mutant leaf edge (Fig. 3D). Also, the abnormal presence of basal cells and macrohairs (Fig. 3D) near the *ns* mutant leaf edge indicates the deletion of a blade domain that includes the margin. In wild-type leaves these structures are located in more laterally median regions, not at leaf margins (Sharman, 1942). Wild type and *ns* leaf edges are indistinguishable distal to the mid-length of the blade (Fig. 3A and B).

The *ns* mutant phenotype shows duplicate factor inheritance

Self-pollinated plants obtained via the outcross of *ns* mutant plants onto nonmutant, inbred stocks segregated the *ns* mutant phenotype in a ratio of 1:15 (Table 1). These results indicate that in most lines, *ns* is an inherited trait specified by two, unlinked mutations. The only exception to this 1:15 F_2 ratio was in the original *ns* 1:1 stock, wherein the mutant phenotype displays

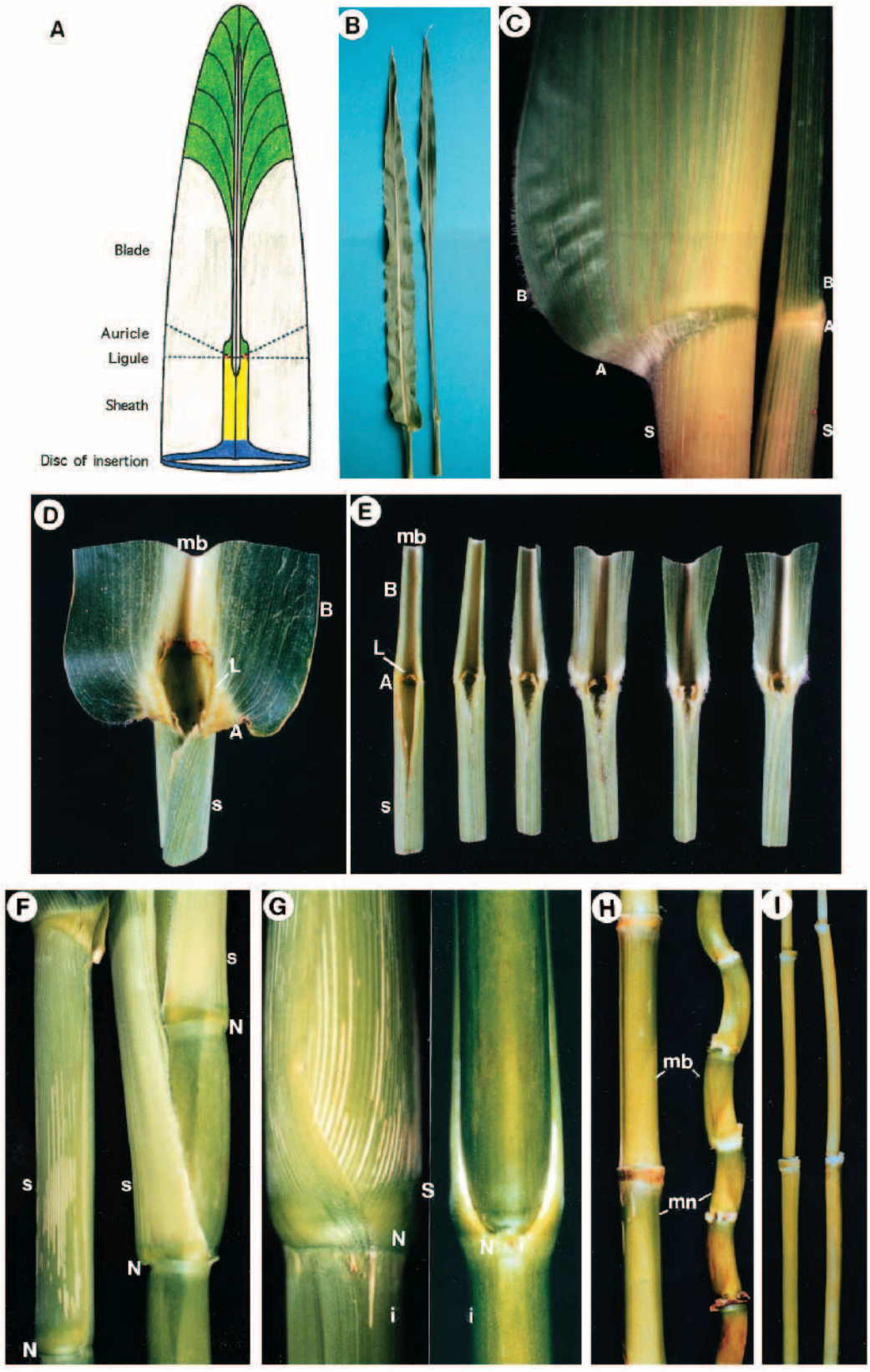

Fig. 2. Morphology of *ns* mutant leaves. (A) Schematic diagram illustrating the major components of the maize leaf. Regions of leaf present in nonmutant siblings but deleted from *ns* mutants are colored gray, and include large portions of the blade (green), auricle (red), and sheath (yellow). (B) The width of *ns* mutant leaves (right) is extremely reduced proximal to the mid length of the blade. The length of *ns* mutant leaves is comparable to nonmutant siblings (left). (C) Side view close-up of the blade-sheath junction in nonmutant sibling (left) and *ns* mutant (right) leaves illustrating the great reduction in blade (B) and auricle (A) width, and in the number of lateral veins (stained red) in *ns* mutants. Close-up of the adaxial surface of nonmutant sibling leaf no. 15 (D) and (left to right) *ns* mutant leaves no. 13 through no. 18 (E). Note the gradual alleviation of the leaf deletion phenotype seen in each successive mutant leaf. (F) Side view close-up of the sheath regions of *ns* mutant (right) and nonmutant sibling (left) leaves illustrating the vast reduction in *ns* mutant sheath (S) width and internode length. (G) Close-up of nonmutant sibling (left) and *ns* mutant lower sheath (S), node (N) and internode (i). Note that the disc of leaf insertion at the node (N) completely encircles the stem in both nonmutant and *ns* mutant leaves. (H) Stem segments from the basal nodes of nonmutant sibling (left) and *ns* mutant (right) plants. Leaves have been removed at the nodes to reveal the internodes. Note that the sides of the internodes corresponding to the leaf margin (mn) of *ns* plants are shorter than the sides corresponding to the leaf midrib (mb), resulting in curved, mutant stems. (I) Although the *ns* mutant (right) internode lengths are normal in upper internodes, they remain curved as compared to wild type (left).

single factor inheritance (Table 2). These data suggest that the *ns* progenitor was identified in a line that previously was homozygous for one of the *ns* mutations. The *ns* loci were located to chromosome arms 3S (*ns1*) and 4L (*ns2*) (Scanlon and Freeling, 1995) using TB translocation stocks (Roman and Ulstrup, 1951; Beckett, 1978). Dosage studies using mosaic aneuploid plants demonstrate that the *ns* mutant alleles behave as loss of function mutations (M. Scanlon and M. Freeling, unpublished results).

The *ns* mutations disrupt primordial events in leaf morphogenesis

SEM of *ns* mutant and sibling nonmutant leaf replicas was performed to help determine the timing of the developmental lesion in *ns* mutant leaves. Although the bases of both nonmutant and *ns* mutant primordia are tubular, the edges of mutant primordia fail to enclose the shoot apex (Fig. 4). This reduction in primordial leaf width forms a 'window' in the [P3] mutant primordium that exposes the shoot apex (Fig. 4D). The *ns* 'window ' persists in later stages of leaf primordial development (Fig. 4F) and is discernible as early as [P2] (Fig. 4B). When followed from tip to base, the edge of the nonmutant [P2] primordium forms a curve with a relatively smooth downward slope (Fig. 4A). The curved edge of the *ns* [P2] primordium, however, is markedly steeper in an area that predicts the eventual formation of the '[P3] window' (Fig. 4B). Thus, leaf development in the *ns* mutant is altered very early, preceding the [P2] primordial stage.

Sylvester et al. (1990), used SEM to measure the depths of

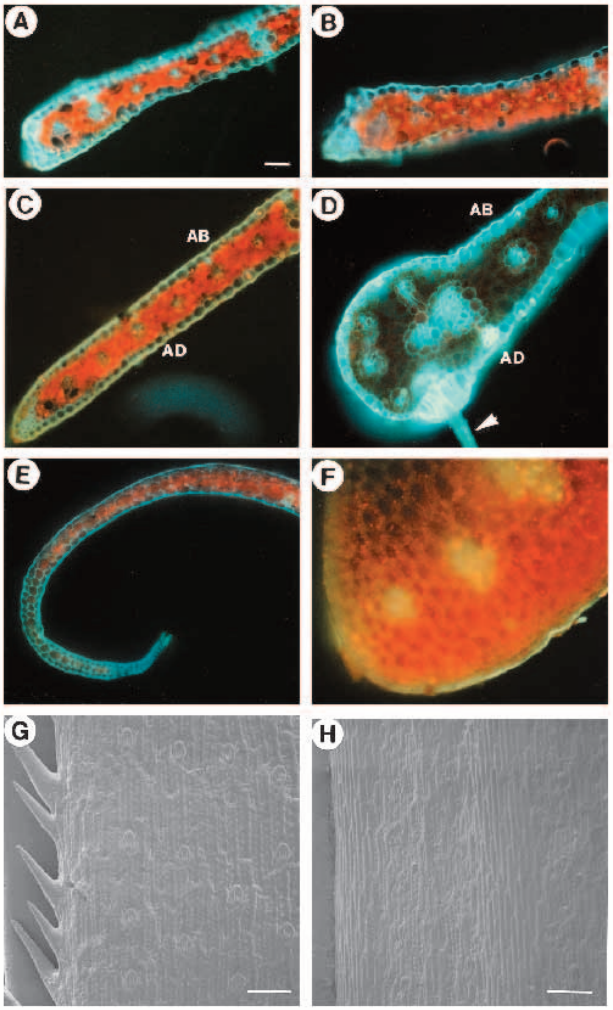

Fig. 3. A margin domain is deleted in *ns* mutants. Fluorescence microscopy of free-hand sections of the edges of nonmutant sibling (A,C,E and G) and *ns* mutant (B,D,F and H) leaves. Although the edges of nonmutant (A) and *ns* mutant (B) leaves are indistinguishable distal to the mid length of the blade, the tapered margins found in the nonmutant proximal blade (C) and sheath (E) are deleted in *ns* mutants (D,F). Red indicates the presence of chlorophyll; the leaf epidermis and veins are blue/white. (G,H). SEM of leaf edge replicas from the proximal region of the blade demonstrate the deletion of saw tooth hairs in *ns* mutant leaves. Arrow in D indicates a basal cell hair. AB, abaxial leaf surface; AD, adaxial leaf surface. Scale bars, (A-F) 160 μm; (G,H), 83.3 μm.

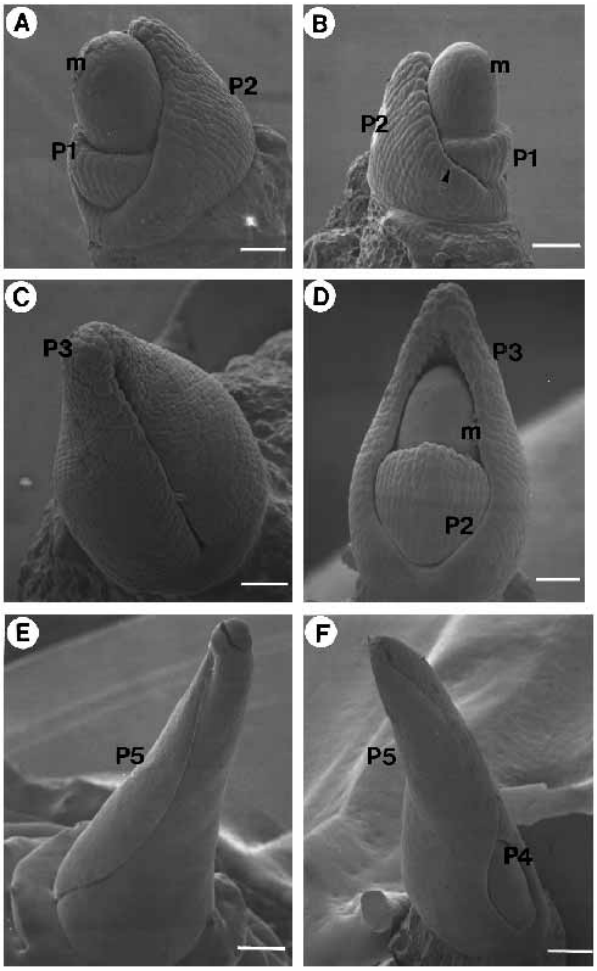

Fig. 4. SEM replica analyses of nonmutant sibling (A,C,E) and *ns* mutant (B,D,F) leaf primordia reveal that the deletion of a margin domain results in a detectable alteration in the shape of the edge (arrowhead) of the mutant [P2] primordium (B) as compared to nonmutant siblings (A). The domain deletion results in a 'window' that forms during the [P3] stage of *ns* mutant leaf primordial development (D) and persists beyond the [P5] (F) stage. The edges of nonmutant [P3] (C) and [P5] (E) leaf primordia overlap and encircle the shoot apex. Note that the leaf bases in both mutant and nonmutant samples are tubular. The node derives from the upper part of this disc of insertion, whereas the internode forms from the lower region (Sharman, 1942). Scale bars, (A,B) 37 μm. (C,D) 35 μm. (E,F) 170 μm.

Table 1. Progeny obtained from self-pollination of ns/Mo17 and ns/B73 heterozygous plants

F_1 genotype	Number of mutant plants	Number of normal plants	Total number of plants	% mutant plants
ns/Mo17	66	849	915	7.21
ns/B73	68	1153	1221	5.57
Total	134	2002	2136	6.27

$\chi^2 = 0.001$ Fits a 15:1 ratio.

cellular crosswalls and quantify cell division indices in developing maize leaf primordia. The results of this study supported the conclusions of previous clonal analysis (Poethig, 1984) which indicated that during primordial growth, all founder cell derivatives divide equally to effect a rapid, expansive growth. Using the technique of Sylvester (1990), the cell division indices near the edges of [P3] leaf primordia of *ns* mutants and nonmutant siblings were measured. The fraction of cells having undergone recent cell division (as indicated by shallow intercellular crosswalls) was found to be 0.638 in *ns* [P3] primordia and 0.642 in wild-type siblings.

ns mutants exhibit altered homeodomain gene expression in the shoot apical meristem

Previous work by Smith et al. (1992, 1995) and Jackson et al. (1994) showed that expression of the *knotted1* homeobox gene may serve as useful marker of leaf/non-leaf boundaries in the vegetative shoot apical meristem. A polyclonal antibody to the KN1-like homeodomain proteins (KNOX; Kerstetter et al., 1994) was used to compare the accumulation pattern of KNOX proteins in *ns* meristems compared to wild-type siblings (see Materials and Methods and Fig. 1 for KNOX antibody preparation). The results show that KN1 down regulation in the founder cell ribbon is subtly yet distinctly different from that of nonmutant siblings (Fig. 5). Transverse sections of nonmutant meristems reveal a doughnut-shaped ring of putative [P0], preprimordial cells which down regulate KNOX accumulation (Fig. 5A). In *ns* mutants this ring is reduced to a crescent (Fig. 5C). In longitudinal sections of nonmutant meristems downregulation of KNOX accumulation is seen in a large patch of cells in the pre-midrib domain, and in a smaller patch of cells on the opposite flank of the meristem corresponding to the pre-marginal region of the incipient leaf (Fig. 5D and F). In *ns* mutants, although KNOX down regulation in the pre-midrib region of the incipient leaf is similar to that of nonmutant siblings, the pre-marginal region does not exhibit KNOX down regulation (Fig. 5E and G). All cells on the flank of the meristem opposite the pre-midrib of the incipient leaf show high levels of accumulation of KNOX proteins. In serial sections through the meristems of all eighteen *ns* seedlings examined, the incipient leaf founder cells of *ns* mutants failed to encircle the apex. Approximately 18-30 cells occupying the presumed pre-marginal region of the mutant [P0] do not show down regulated KNOX accumulation (Fig. 5). Despite this alteration in KNOX accumulation, the histology of *ns* meristems is indistinguishable from nonmutant siblings. Moreover, cell counting comparisons in the lateral, transverse, and longitudinal dimensions have revealed no significant differences in cell number between *ns* mutant and nonmutant meristems.

Table 2. Progeny obtained from self-pollination of normal plants generated from cross of ns mutant by non-mutant sibling plants in original stock obtained from Pioneer Hi-Bred International

Sample number	Number of mutant plants	Number of normal plants	Total number of plants	% mutant plants
1	24	62	86	27.9
2	14	40	54	25.9
3	7	29	36	19.4
4	48	150	198	24.2
5	8	25	33	24.2
6	12	21	33	36.4
7	22	78	100	22.2
Total	135	406	541	24.9

$\chi^2 = 0.001$ Fits a 3:1 ratio.

DISCUSSION

The *ns* mutant exhibits a leaf deletion phenotype which removes marginal structures and approximately one-half of the lateral veins. The putative domain deleted in *ns* leaves does not encompass the entire length of the mutant leaf, but includes the whole of the sheath and the proximal half of the blade. One feature of this margin domain is a tapered leaf edge. The extent of this domain also mimics that of x-ray-induced, genetically mosaic sectors encompassing the leaf margin (Steffenson, 1968; Poethig 1995, 1984, and M. Scanlon, personal observations), which indicates that the cells in this region are clonally related.

Sharman (1942) showed that the complete complement of lateral veins form during the primordial stage of maize leaf development. Therefore, the reduced number of lateral veins in *ns* mutants indicates a preprimordial developmental block. In addition, measurements of cell division indices of [P2] and [P3] leaf epidermis revealed no meaningful differences in *ns* mutant and nonmutant primordia. An important implication of these data is that the *ns* mutations do not simply retard the expansive, primordial growth of leaf founder cells initialized to form the leaf margins. Instead, these observations indicate that the *ns* lesion precedes the primordial stage of proliferative leaf growth and may altogether eliminate a leaf margin domain.

A feature found in many maize mutants with narrow leaves is a smaller-sized, often misshapen meristem (M. Scanlon, F. Baker and M. Freeling, unpublished results). In *ns* mutants however, the meristem is not morphologically aberrant. The only variation detected in *ns* mutant meristems is a subtle change in the leaf/non-leaf boundary as indicated by KNOX accumulation. A small subset of founder cells corresponding to the pre-marginal domain accumulate KNOX proteins at a developmental timepoint when the adjacent founder cells do not. We suggest that the margins are deleted in *ns* mutant leaves because this region of the preprimordial developmental field is never initialized from the meristem, and is thereby excluded from the developing leaf. In short, the leaf margin meristematic founder cells exist, but do not 'know' they are leaf.

Genetically mosaic plants containing aneuploid sectors of mutant tissue (*ns1/–* ; *ns2/–*) produce narrow leaves and bent internodes only when the mutant sector includes the marginal flank of the phytomer; sectors of *ns* mutant passing through the midrib regions of the leaf have no developmental phenotype (M. Scanlon and M. Freeling, unpublished data). These results

indicate that the region of action of *Ns* genes does not include the central leaf domains but is restricted to the marginal domains of the phytomer.

Perhaps the *ns* mutations perturb the developmental instructions of a small subset of meristematic cells which are thereby omitted from the founder cell ribbon. Although the altered KNOX accumulation in *ns* meristems is limited to 18-30 of approximately 200 founder cells, more than one-half of the mature leaf is deleted in *ns* mutants (Fig. 2B). However, this apparent incongruity is predicted from clonal analyses of maize that revealed increased growth rates near leaf margins during post-primordial growth (Steffenson, 1968; Poethig, 1984; Poethig and Szymkowick, 1995).

In light of the data presented, a model of *Ns* gene function is presented in Fig. 6. We propose that the *Ns* gene products initialize cells in a specific domain of the meristem to become leaf founder cells. This model presupposes that preprimordial leaf development proceeds via at least two separate recruitment stages. In the first step, previously naive meristematic cells are initialized to assume leaf founder cell identity. As diagrammed in Fig. 6, this process begins in the flank of the meristem which will form the midrib region of the leaf, and progresses toward the pre-marginal flank of the meristem. Once initialized, the founder cells are competent to respond to a second round of meristematic signaling, wherein leaf domain-specific fates (indicated as color-coded lateral segments in Fig. 6) are assigned.

In this view, the *Ns* loci act during the first stage of founder cell recruitment to initialize cells in the marginal flank of the meristem (opposite the flank wherein leaf initialization begins) (Fig. 6B). This model says that *Ns* gene activity is blind to the specific fate of the meristematic cells it serves to initialize; the domain-specification signal(s) acts later in preprimordial development. Instead, the *Ns* gene products act in the marginal flank of the meristem to tell those cells to become leaf, and equip them to respond to a later, domain-specification signal. Thus, lack of founder cell initialization in the pre-marginal domain of the *ns* mutant meristem causes a failure of those cells to respond to a leaf domain-differentiation signal, resulting in the deletion of those regions from the developing leaf.

According to this model, as the meristem enlarges with every plastochron (Bassiri et al., 1992), progressively less and less of the founder cell ribbon will pass into the uninitialized patch of the *ns* mutant meristem (Fig. 6C). Fewer leaf domains are thereby encompassed by the *ns* meristematic lesion, resulting in an alleviation of the deletion phenotype in leaves produced by the older, larger vegetative meristem. The alleviation would be

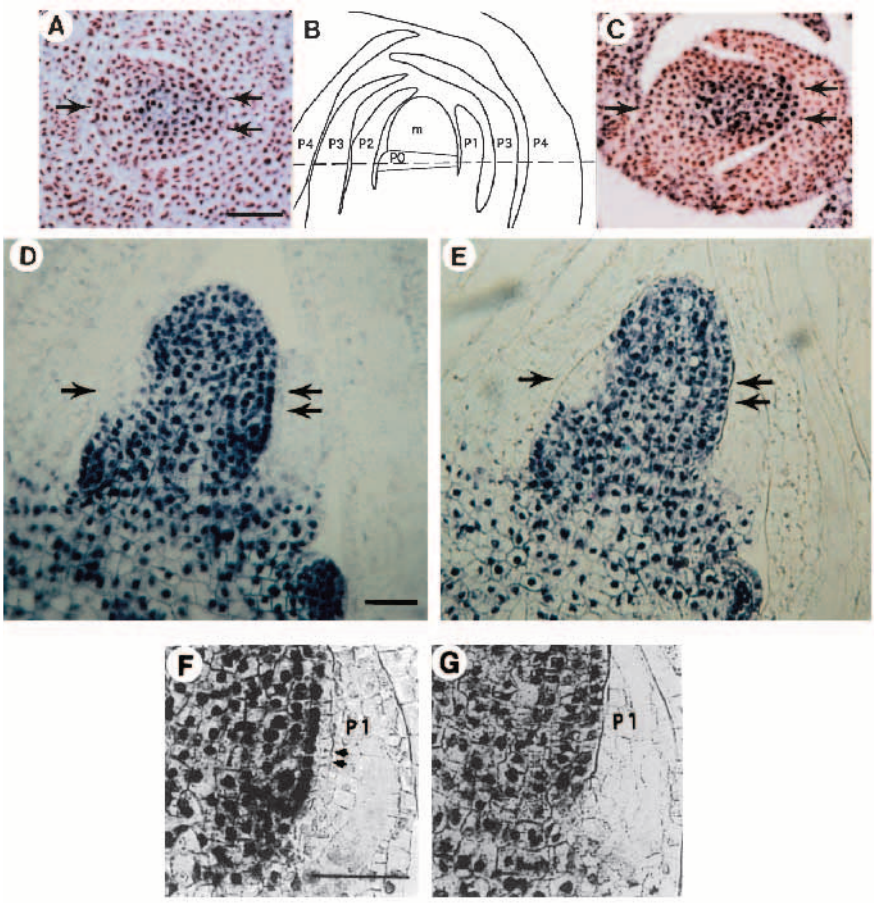

Fig. 5. *ns* mutants exhibit a subtle alteration in the accumulation pattern of homeodomain proteins. Immunohistochemical analyses of the accumulation of the KNOX maize homeodomain proteins were performed on nonmutant (A,D,F) and *ns* mutant (C,E,G) 14-day-old vegetative shoot apices. The schematic drawing (B) illustrates the arrangement of leaf primordia surrounding the vegetative shoot meristem as seen in median longitudinal sections of the maize shoot apex. The dashed line passes through the meristem (m) at the level of the incipient leaf founder cells and illustrates the approximate region of transverse sections through the apices shown in A and C. (A) In transverse sections of wild-type apices, KNOX accumulation (dark blue nuclei) is localized to the meristem proper and is down regulated (pink nuclei) in leaf primordia and in a ring of cells surrounding the meristem whose position and histology indicate them to be the incipient leaf pre-primordium [P0]. (C) In transverse sections of *ns* mutant shoot apices, KNOX downregulation (pink nuclei) in the pre-midrib region (single arrow) of the incipient leaf is similar to that of nonmutant siblings. However, the pre-marginal region (double arrows, a single-cell deep ribbon of epidermal meristem directly opposite the premidrib), exhibits KNOX accumulation (black nuclei) in mutant meristems only, such that the incipient leaf founder cells of *ns* mutants fail to encircle the apex. (D) In median longitudinal sections of nonmutant apices the founder cell ring is visualized as patches of KNOX downregulation (unstained nuclei) on the flanks of the meristem corresponding to the pre-midrib (single arrow), and pre-marginal (double arrows) domains of the incipient leaf. (E) Longitudinal sections of *ns* mutant meristems reveal subtle patches of KNOX accumulation in the pre-marginal region of the [P0] (double arrows). (F,G) Close-up of the pre-marginal domains of nonmutant (F) and *ns* mutant (G) leaf founder cells. A region of KNOX downregulation is observed in the tunicate cell layer of the nonmutant meristem. This subtle region extends 3-4 cells high and one cell deep (arrows). The small size of these tunicate cells and the orientation of their cell walls indicate recent periclinal divisions diagnostic of incipient leaf cells (Ledin, 1954). Epidermal cell divisions in the pre-marginal region are also evident in transverse sections of nonmutant meristems (A) but not in the pre-marginal domains of *ns* mutant meristems (C,E,G). Scale bars, 500 μm.

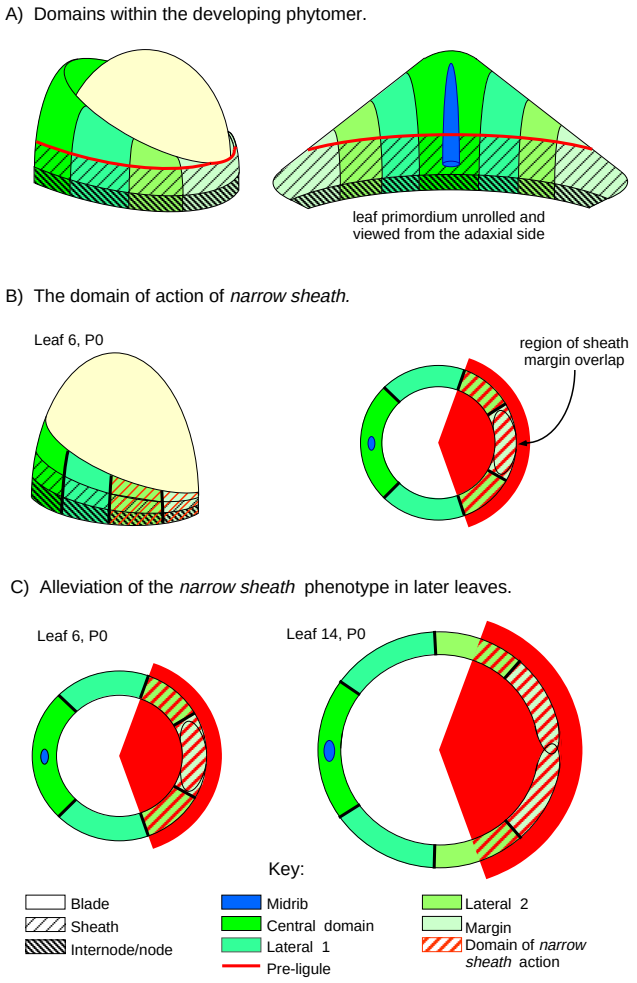

Fig. 6. Model of *Ns* genes function. (A) Schematic representation of the maize vegetative meristem and leaf primordium. Several hypothetical leaf domains are represented as lateral segments (see legend) each of which is divided into a proximal node/internode region, middle sheath region and a distal blade region. (B) The domain of *Ns* genes is modeled as a sector (red) of the shoot apical meristem. On the left is a meristem and founder cell ribbon; on the right is a transverse section through the meristem at the level of the incipient leaf. The arrow indicates the overlapping left and right sheath margin domains. The *Ns* genes act to initialize previously naive cells in a specific quadrant of the shoot apical meristem. Once initialized, these cells are competent to respond to later signals that specify a particular leaf domain identity. This model predicts that the *Ns* signal acts before domain-specific fates are assigned to the leaf initials. In *ns* mutants, meristematic cells in this region remain uninitialized, and therefore cannot respond to the later signals specifying domain identities. (C) The alleviation of the *narrow sheath* phenotype in later leaves is explained in terms of the model. Schematic representation of meristematic leaf founder cell ribbons [P0] from leaf 6 (left) and leaf 14 (right). Because the meristem enlarges with each successive plastochron (Bassiri et al., 1992), it is predicted that the leaf founder cell ribbons exhibit less overlap in later leaves. If the *Ns* genes function in a specific quadrant of the meristem, a smaller percentage of the leaf founder cells are initialized by the *Ns* genes in leaf 14 than in leaf 6. In *ns* mutants, loss of *ns* gene function would result in lack of initialization of a larger fraction of the founder cell ribbon in leaf 6 than in leaf 14. In turn, this results in a larger leaf deletion phenotype in earlier versus later leaves of *ns* mutants (see Fig. 1E).

expected to be most extreme in the uppermost leaves of the plant, because these leaves are smaller (fewer founder cells) and formed from a larger meristem (Bassiri et al., 1992) than more basal leaves. This syndrome is observed in *ns* mutant plants. If however, (1) the initialization of meristematic cells to become founder cells and (2) the assignment of domain-specific identities were both accomplished in a single step, the alleviation phenotype would not be predicted unless the *Ns* genes specified different leaf domains in different leaves.

The maize leaf, node and internode are terms used to delineate clonally related portions of the same phytomer (Galinat, 1959; Poethig and Szymkowick, 1995). In maize, the extreme lower leaf zone forms a tubular base. The upper portion of this leaf base is the node, whereas the lower portion comprises the internode (see Fig. 4; Kaplan, 1973; Sharman, 1942). Although the disc of leaf insertion at the node is not noticeably affected in *ns* mutants (Fig. 2F,G) the marginal side of the internode is shortened, forming sinuous stems (Fig. 2H,I). These data are consistent with our model describing the role of the *ns* gene products as initializing meristematic cells to assume leaf founder cell identity (Fig. 6B). In the internode, lack of pre-marginal founder cell initialization deletes a slice from one side of the tubular leaf base. Following longitudinal growth, the result of the *ns* deletion is curved stems. In contrast to internodes and the leaf proper, the node does not undergo extensive longitudinal expansion during development. Therefore, the *ns* domain deletion is not as phenotypically obvious in the node as in the internode or the leaf proper.

The asymmetric leaf width phenotype seen in upper leaves of *ns* mutants also supports our two-stage model for leaf founder cell recruitment. Clonal analyses of maize leaves indicate that the midrib axis often varies in position from leaf to leaf (Steffenson, 1968; M. Scanlon, personal observations). That is, the midrib axes in successive leaves frequently do not line up 180 degrees apart; there are often small shifts in the axis of leaf initiation. According to our model, during the first stage of preprimordial leaf development meristematic cells are initialized and made competent to respond to leaf domain specifying signals. The midrib axis is therefore the first leaf domain identity specified during the second stage of founder cell recruitment (Fig. 6). Soon after, the central domain, the lateral domains, and finally the marginal domain identities of the founder cell ribbon are specified in succession. Our model ascribes that the *Ns* genes initialize cells in a flank of the meristem during the first stage of founder cell recruitment, and are blind to the domain-specific fate assigned in stage two (Fig. 6B). In *ns* mutants however, cells in the *ns* region cannot respond to domain specification signals and are excluded from the leaf. A midrib axis shift at the beginning of stage two would therefore cause unequal portions of left and right lateral founder cell domains to fall into the uninitialized, *ns* zone of the meristem. This would result in an unequal deletion of leaf domains on the left versus the right side of the leaf, and cause leaf width asymmetry. As with the alleviation phenotype discussed previously, the leaf asymmetry phenotype is not easily explained by a model wherein founder cell initialization and assignment of domain-specific identity are executed in a single step.

Studies of several plant homeotic mutations which transform one organ into another have begun to elucidate processes whereby identity is established and maintained in a developmental field (Bowman et al., 1989; reviewed by Coen and

Meyerowitz, 1991; Sakai et al., 1995). These studies indicate that the leaf is a ground state organ in plants; floral organs are derived via additive modifications of the basic leaf design. Previous studies have argued that developmental compartments exist within plant as well as animal organs (Jürgens et al., 1991; Dawe and Freeling, 1992; Cerioli et al., 1994). Our results suggest that patterning of plant organs into semi-independent domains may occur in the meristem. In this model, the *ns* mutant represents a preprimordial domain deletion in the founder cells of a plant, ground state organ. Studies of pattern formation in *Drosophila* were enhanced by phenotypic analyses of segment deletion mutants (Nüsslein-Volhard and Wieschaus, 1980; reviewed by Wilkins, 1993). Our initial characterization of the domain deletion mutant *ns* represents an early step toward understanding the processes whereby meristematic cells are initialized to form one of several hypothesized leaf domains. Similarities between compartmentalized wing formation in *Drosophila* and 'patchwork' leaf development in maize are alluring, especially considering the separate evolution of multicellular form by plants and animals (reviewed by Avers, 1989). Further genetic analyses of leaf development utilizing pattern deletion mutants like *ns* can assess the relevance of these similarities.

The authors thank L. Smith and S. Hake for kindly providing the KN1 affinity matrix and KN1 antiserum; M. Mooney for providing the drawings in Fig. 2A and Fig. 6, and for helpful discussions of the data; F. Baker for the sample used in Fig. 4E; W. Bentham and the U.C. Berkeley Electron Microscopy Lab for technical advice and assistance; M. Mooney, L. Harper, G. Muehlbauer and R. Tyers for critical reading of the manuscript; and D. J. Houghton for assistance in and out of the field. This work is supported by a NIH grant 2 ROI GM42610-06 to M.F.; M.S. is supported by NSF postdoctoral fellowship BIR-9303608; R.S is supported by a NIH postdoctoral fellowship F32GM14578-02.

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(Accepted 14 March 1996)