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The *rough sheath2* gene negatively regulates homeobox gene expression during maize leaf development

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SUMMARY

Leaves of higher plants are produced in a sequential manner through the differentiation of cells that are derived from the shoot apical meristem. Current evidence suggests that this transition from meristematic to leaf cell fate requires the down-regulation of *knotted1*-like homeobox (*knox*) gene expression. If *knox* gene expression is not repressed, overall leaf shape and cellular differentiation within the leaf are perturbed. In order to identify genes that are required for the aquisition of leaf cell fates, we have genetically screened for recessive mutations that confer phenotypes similar to dominant mutations (e.g. *Knotted1* and *Rough sheath1*) that result in the ectopic expression of class I *knox* genes. Independently derived mutations at the *rough sheath2* (*rs2*) locus condition a range of pleiotropic leaf, node and internode phenotypes that are sensitive to genetic background and environment. Phenotypes include dwarfism, leaf twisting, disorganized differentiation of the blade-sheath boundary, aberrant vascular patterning and the generation of semi-bladeless leaves. *knox* genes are initially repressed in *rs2* mutants as leaf founder cells are recruited in the meristem. However, this repression is often incomplete and is not maintained as the leaf progresses through developement. Expression studies indicate that three *knox* genes are ectopically or over-expressed in developing primordia and in mature leaves. We therefore propose that the *rs2* gene product acts to repress *knox* gene expression (either directly or indirectly) and that *rs2* gene action is essential for the elaboration of normal leaf morphology.

Key words: Maize, Leaf development, Homeobox, *rough sheath2*

INTRODUCTION

A unifying feature of higher plant development is the sequential initiation of lateral organs from the shoot apex. Leaf development in particular marks a fundamental change in the behaviour of cells at the apex, however, factors that regulate the timing, position and size of leaf primordia initiated from the apex are largely unknown. Maize initiates leaves in a distichous pattern by the recruitment of cells on the flanks of the shoot apex into a ribbon-like primordium of cells that eventually encircles the apex in an overlapping ring. These cells are termed the 'disk of insertion' or founder cells and have been defined by histological, clonal, and molecular criteria (Sharman, 1942; Poethig, 1984; Smith et al., 1992; Scanlon and Freeling, 1997). The first histologically visible signs of leaf development occur when densely cytoplasmic founder cells on the periphery of the meristem begin to divide rapidly in both the L1 and L2 layers. Cell divisions then spread around the circumference of the apex, in both directions from the initiation point, until a young primordium is evident encircling the apex (Sharman, 1942; Sylvester et al., 1990). Fate mapping studies demonstrate that the founder cell population is composed of approximately 200 cells in a 2-3 cell high tier that is about 30 cells in circumference and occupies at least two cell layers (Poethig and Szymkowiak, 1995). In addition to forming the main parts of the leaf (blade, sheath, auricle and ligule), the founder cells also give rise to both the subtending node and internode of the culm (Sharman, 1942; Johri and Coe, 1983; Poethig and Szymkowiak, 1995).

Although meristems from different species vary widely in form, they share a number of common properties with respect to the leaf initiation process. For example, a redirection of cellular growth on the periphery of the shoot apex in the founder cell ribbon leads to the formation of a leaf buttress or a protrusion of cells outward from the apex (Sharman, 1942; Steeves and Sussex, 1989). Other features common to leaf initiation in many flowering plants are an increase in cell division indices and the down-regulation of *knotted1*-like homeobox (*knox*) gene expression (Sharman, 1942; Smith et al., 1992; Jackson et al., 1994; Lincoln et al., 1994; Ma et al., 1994; Kerstetter et al., 1995; Schneeberger et al., 1995; Long et al., 1996; Hareven et al., 1996). In maize, the *knotted1* (*kn1*) homeobox gene is expressed in the central zone of the shoot apex but not in the peripheral zone where leaf initiation is

occurring (Smith et al., 1992; Jackson et al., 1994). Other *knox* genes in maize are also repressed in leaf primordia but each adopts a specific expression pattern within the developing phytomer. For example the *rough sheath1* (*rs1*) gene is expressed in a donut shaped ring underneath leaf primordia (Schneeberger et al., 1995).

Studies of both dominant *knox* mutants and of transgenic lines overexpressing *knox* genes, indicate that leaf development in both monocots and dicots is very sensitive to the ectopic expression of *knox* genes (Smith et al., 1992; Sinha et al., 1993; Schneeberger et al., 1995; Lincoln et al., 1994; Muller et al., 1995; Hareven et al., 1996; Fowler et al., 1996; Muehlbauer et al., 1997; Williams-Carrier et al., 1997). In general, the ectopic expression of *knox* genes in leaf primordia causes a retardation of leaf development such that cells in distal regions adopt a more basal cell fate (Freeling, 1992; Sylvester et al., 1996; Muehlbauer et al., 1997). This transformation to a more basal cell fate can be extreme in dicots, resulting in the production of leaf-born shoots (Chuck et al., 1996). In tomato, leaf shape is also perturbed when *knox* genes are ectopically expressed in leaves. In these overexpressing lines, leaves are 'super compound' and essentially lack blade lamina (Hareven et al., 1996; Chen et al., 1997). These results strongly suggest that *knox* genes are important factors in determining the size and shape of leaves in higher plants.

Recent studies with other genes that are involved in leaf formation and meristem function also suggest that the down-regulation of homeobox genes is necessary to signal the switch between a relatively indeterminate non-leaf state and commitment to a leaf development program. For example, mutations at the duplicate factor *narrow sheath* (*ns*) loci cause the formation of exceptionally narrow leaves. The mutant phenotype is associated with the failure to down regulate *knox* gene expression in a small group of approximately 18 cells on the premargin side of the phytomer (Scanlon et al., 1996). Significantly, clonal analysis of leaf development in *ns* mutants demonstrates that these cells are not incorporated into the leaf primordium and thus do not contribute to blade and internode formation (Scanlon and Freeling, 1997).

All of the studies described above suggest that the down-regulation of *knox* gene expression is essential for normal leaf initiation and development. In order to further examine the role of homeobox genes in leaf development and to identify factors involved in their regulation, we have genetically screened for recessive mutations that mimic the phenotype produced by the ectopic expression of *knox* genes in leaf primordia (Freeling and Hake, 1985; Becraft and Freeling, 1994; Fowler and Freeling, 1996). This report describes our analysis of maize *rough sheath2* (*rs2*) mutants. Our results show that *rs2* plays an important regulatory role in leaf initiation through its activity on *knox* gene expression.

MATERIALS AND METHODS

Maize stocks and growth conditions

The *rs2* reference (*rs2-R*) allele (isolated by M.I. Hajidov in 1937) was obtained from the Maize Cooperative Stock Center (Columbia, Missouri) and introgressed five times into B73, W22, W23 and Mo17 inbred lines. The *rs2-twisted dwarf (rs2-twd)* allele was isolated in a transposon mutagenesis experiment that used *Spm* as the mutagen. Complementation tests between *rs2-R* and *rs2-twd* were accomplished by intercrossing heterozygotes and screening the F_1 progeny for mutant individuals. In all cases, one quarter of the progeny were mutant. Lines carrying an *Sn* allele that controls anthocyanin pigmentation of the sheath, auricle and ligule were a gift from Dr Maria Moreno (Yale University).

Plants for genetic crosses were grown to maturity in the field in Berkeley, CA or in New Haven, CT. For scanning electron microscopy (SEM), histological analyses and RNA analyses, plants were grown in the greenhouse with an average daytime temperature of 32°C and a diurnal cycle of 16 hours light (average 300 μmol/m^2/second[1]) and 8 hours dark.

Microscopy

Samples for scanning electron microscopy (SEM) were either prepared using dental impression medium or critical point dried. Dental impression medium replicas were prepared without fixation as described by Sylvester et al. (1990). For critical point drying, tissue was dissected and immediately fixed for 48 hours in formalin:acetic acid:alcohol:Triton X-100 (3.7%:5%:50%:0%) and then dehydrated to 100% ethanol. Tissue was critical point dried in a Tousimis samdri-pvt-3B critical point dryer. Both replicas and critical point dried material were coated with 25 nm platinum using a Polaron E5400 high resolution sputter coater. Subsequent analysis was carried out on a ISI DS-130 SEM with a LaB6 filament operating at 10 kV.

For light microscopy, samples were either sectioned freehand without fixation or were fixed in formalin:acetic acid:ethanol (10%:5%:50%) and embedded in wax as described by Langdale (1994). Wax embedded samples were sectioned using a rotary microtome. Sections were examined and photographed using either a Zeiss Axiophot or a Leica DMRB microscope.

Immunohistochemistry and RNA in situ localization

Immunolocalization of KNOX proteins was carried out as described by Donlin et al. (1995) using anti-KNOX and anti-RS1 antibodies (Scanlon et al., 1996) and paraffin embedded tissue. RNA in situ hybridizations were performed as previously described by Langdale (1994) using ^{35}S-labeled riboprobes and paraffin embedded tissue.

knox gene transcript analysis

RNA was isolated from immature leaves according to the method of Kloeckener-Gruissem (1992). cDNA was subsequently prepared using Superscript reverse transcriptase (Gibco/BRL) and was used as a template for Polymerase Chain Reaction (PCR) amplification of *knox* genes as described by Bauer et al. (1994). Gene-specific primers used for amplification were as follows:

rough sheath1 a – 5′gagaactacaagccatgcatagacgctac3′
b – 5′ ttctgaagatgacatggacccgaatggtc3′
knotted1 a – 5′agtagtagtgtgggtgcgagatg3′
b – 5′gagatcacccaacactttgg3′
liguleless3 a – 5′gtggaacacgcactaccgctg3′
b – 5′agtggtgtatgattcagggtcc3′.

Control reactions were carried out using primers homologous to a maize ubiquitin gene:

ubi 3 – 5′taagctgccgatgtgcctgcgtcg3′
ubi 4 – 5′ctgaaagacagaacataatgagcacaggc3′.

kn1 primers were a generous gift from T. Foster and S. Hake (USDA, Albany, CA).

RESULTS

rs2 mutant phenotype

rs2 mutant plants have reduced stature and exhibit aberrant development of leaves, branches, floral organs and the stem (Hajidov, 1937). In addition to the original *rs2* reference (*rs2-*

R) allele, we have isolated an *Spm*-induced allele [*rs2-twisted dwarf* (*rs2-twd*)] from transposon mutagenesis screens. Both alleles confer similar recessive phenotypes. *rs2* mutant plants exhibit a striking dwarf phenotype due to reduced and abnormal growth of internodes that is apparent at the seedling stage and becomes very pronounced at maturity (Fig. 1A,B). Dwarfism as well as the leaf and floral phenotypes discussed below are sensitive to genetic background and environmental conditions. The *rs2-R* allele was introgressed into four different genetic backgrounds (B73, W22, W23 and Mo17) and phenotypic severity was determined in each case. In general, an increase in dwarfism is correlated with increasing severity of leaf and floral phenotypes. However, background-specific differences are apparent. The most notable of these are male sterility and the formation of bladeless leaves (Fig. 1C). *rs2-R* plants introgressed into the B73 inbred background display the rough sheath, twisting and dwarfing phenotypes in 100% of homozygous mutants. Up to 90% of these mutants will also show evidence of leaves with multiple midribs, narrow/bladeless leaves and fused nodes. 100% of mutant plants in the B73 background also show a reduction in the number of male and female florets formed plus the generation of multiple silks per ovule, however, pollen is fertile (Fig. 1K). In contrast, the *rs2-R* allele introgressed in the Mo17 background shows the rough sheath and dwarfing phenotype in 100% of mutant plants but only rarely exhibits narrow/bladeless leaves and fused nodes. 90% of *rs2-R* mutants in the Mo17 background are male and female fertile although the number of florets in both male and female inflorescences is reduced.

The most penetrant and pronounced leaf phenotype, regardless of the genetic background or environment, is a disruption of the blade-sheath boundary due to disorganized cell growth and acropetal ligule displacement. This can be most easily visualized in plants that are doubly mutant for *rs2* and an *Sn* allele that pigments the sheath and auricle (Fig. 1F,G). In weakly expressing mutants, the resultant 'rough sheath' phenotype is localized to the blade-sheath boundary, however, in strongly expressing mutants the entire sheath can appear 'rough' (Fig. 1D,I). Although the sheath can be drastically reduced in length, the blade region generally attains normal length (Fig. 1I).

In addition to showing dwarf and rough sheath phenotypes, the most severe mutant plants also manifest a number of other leaf phenotypes. These include leaves with multiple or excessively large midribs, wider leaves with an increased number of veins compared to wild-type and leaves with reduced amounts of blade lamina (Fig. 1C,D,H,I). Leaves with reduced blade lamina range from those with half to three quarters of the normal blade surface area to those that are bladeless and appear to be composed solely of midrib-like tissue (Fig. 1C,I). Such leaves tend to be restricted to upper nodes on the plant and are seldom observed in the first five nodes. An examination of the site of insertion into the stem at the node reveals that reduced lamina leaves are only partially inserted around the circumference of the stem, suggesting that the reduction in blade area is related to the ontogeny of the leaf from the apex. Bladeless leaves often expose the internodes which are normally masked by the encircling sheath in wild-type plants (Fig. 1C). Often semi-bladeless leaves are the result of folding of the leaf margins in toward the point of leaf origin.

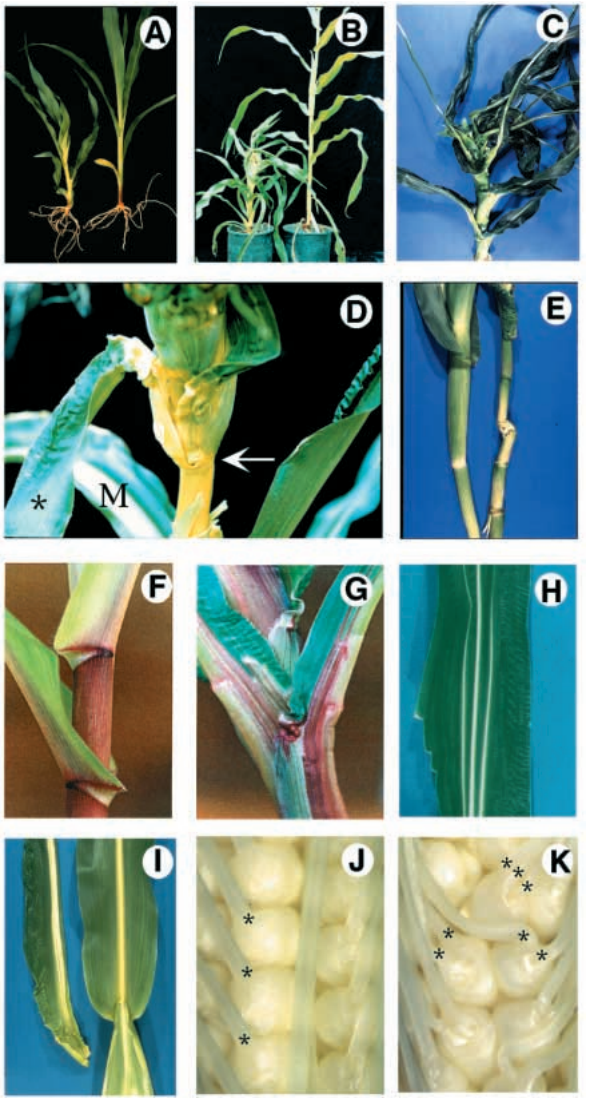

Fig. 1. Phenotype of rs2 mutant plants. (A) *rs2-R* mutant (left) and wild-type seedlings. (B) Mature *rs2-R* mutant (left) and wild-type plants. (C) Severe *rs2-R* mutant exhibiting semi-bladeless leaves. (D) Upper node showing opposite and decussate paired leaves (white arrow). One of the paired leaves lacks a midrib (asterisk). The leaf one node below the paired leaves has an exceptionally large midrib (M). (E) Wild-type (left) and *rs2* (right) culms showing the highly compressed and twisted nature of *rs2* mutant internodes. (F) Wild-type plant carrying *Sn* allele showing purple pigmentation of sheath and auricle. (G) *Sn;rs2-twd* double mutant showing displaced and ill-defined blade sheath boundary. (H) *rs2-R* mutant leaf exhibiting triple midribs. (I) *rs2-R* leaf (left) with half a blade, large midrib and foreshortened sheath compared to wild-type (right). (J) Wild-type carpels each with one silk (pistil) denoted by an asterisk. (K) *rs2* mutant carpels with multiple silks.

The node and internode associated with reduced lamina leaves are also abnormal and display a highly compressed and curved phenotype (Fig. 1E). Occasionally, two nodes will appear to be joined at one side suggesting a fusion of successive leaf nodes. These fused nodes can also take the appearance of opposite and decussate phyllotaxy (Fig. 1D). Leaves initiated at the same node on the same side of the apex are also occasionally observed. In this case, the two leaves show opposite

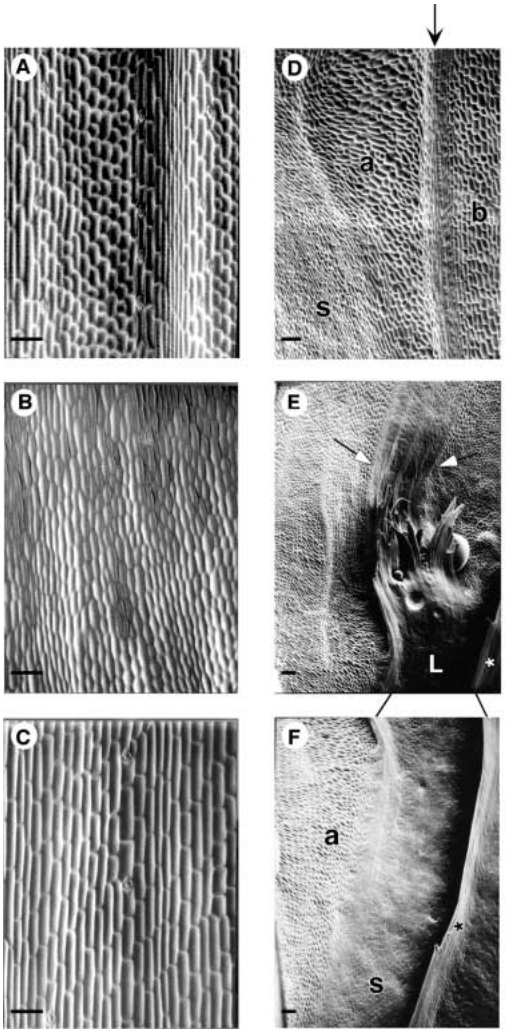

Fig. 2. Cell fate alterations in rs2 leaves. SEM analysis of wild-type and *rs2* epidermal cells. Leaves 6-8 were harvested from 6-week old plants. (A) Wild-type blade. (B) Wild-type auricle. (C) Wild-type sheath. (D) *rs2* leaf showing a sector of auricle and sheath-like cells adjacent to blade-like cells (black arrow marks boundary of blade and auricle phenotypes). (E) *rs2* leaf showing a ridge of ligule cells (marked by the white asterisk) juxtaposed to sheath and auricle cells. Note the cells above the ectopic ligule (L) show a blade cell fate with a sharp boundary (arrows) to the auricle like cells right and sheath like cells left. (F) *rs2* leaf replica taken just proximal to that shown in E. Black asterisk, continuation of ligule ridge shown in E; black lines connecting panel E and F indicate continuation of ligule ridges in adjacent replicas; s, sheath; a, auricle; b, blade. The upper side of each panel corresponds to the distal end of the leaf with respect to the point of origin. Size bar, 200 μm.

orientations of adaxial and abaxial surfaces similar to the ectopic leaves observed in *Lax midrib* mutants and in *knotted1* loss of function mutants (Schichnes et al., 1997; Kerstetter et al., 1997).

In addition to affecting vegetative development, the *rs2* mutation also perturbs floral development. One of the most notable floral phenotypes in the mutant is the production of multiple pistils from the carpel resulting in mature ears that appear exceptionally ‘silky’ (Fig. 1J,K). Significantly, extra

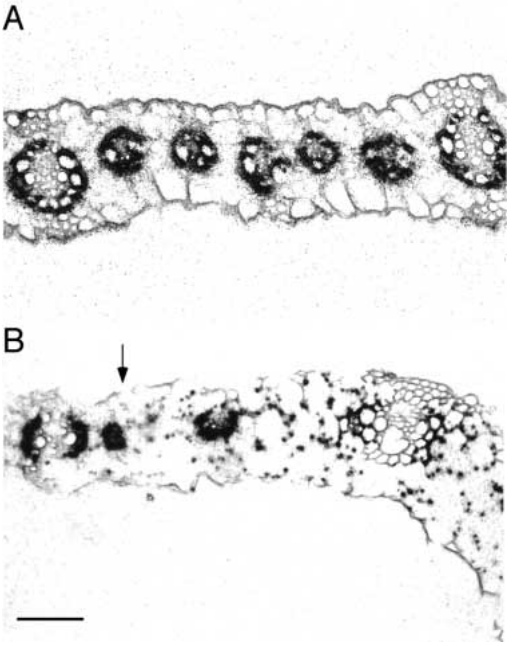

Fig. 3. In situ hybridization of *rbcL* to *rs2-twd* mutant leaves. Sections through a wild-type leaf blade (A) and a *rs2-twd* mutant leaf blade with aberrant vascular spacing (B). Second leaves of 3- week old plants were examined. In regions that display normal vein spacing patterns (A; to the left of the arrow in B), *rbcL* is localized in the bundle sheath cells. However, in regions of the leaf where veins are more than four cells apart (to the right of the arrow in B) *rbcL* accumulates in mesophyll cells. Thus, ‘patches’ of C_3-like tissue can be seen in the leaf. Scale bar, 70 μm.

silk production is also a feature of the dominant mutant *Gnarley1* (*Gn1*) in which the *gn1* homeobox gene is ectopically expressed (T. Foster and S. Hake, personal communication). Staminate flower development in *rs2* mutants is also perturbed in that glumes and lemmas display similar leaf folding phenotypes to those observed during vegetative leaf development (data not shown).

Cellular differentiation in *rs2* mutant leaves

The phenotype of *rs2-twd;Sn* mutants indicated that the rough sheath nature of the mutant phenotype was due to a transformation of leaf blade tissue into sheath tissue. In order to determine the extent to which cell fate transformations occur, we examined cellular differentiation patterns in both L1- and L2-derived tissue of wild-type and mutant leaves. An SEM examination of wild-type leaf surfaces revealed distinct differences in epidermal cell shape in the blade (Fig. 2A), auricle (Fig. 2B) and sheath (Fig. 2C). In leaves of *rs2-R* plants, however, blade-sheath boundaries are ill-defined in that epidermal cells in the blade adopt a mixture of sheath- and auricle-like cell fates (Fig. 2D). The transformed regions frequently appear as sectors running up into the blade with the borders of these sectors often composed of long narrow cells reminiscent of ligule cells (Fig. 2D-F).

In addition to abnormalities in epidermal cell characteristics, *rs2* mutants display abnormal development of subepidermal cells. Transverse sections of wild-type and mutant leaves show that *rs2* leaves are thicker in the basal half due to an excess of

mesophyll cells. Furthermore, increased numbers of mesophyll cells in the transverse dimension produce a vein spacing pattern similar to that seen in the sheath (i.e. more than four cells separate two veins). It has previously been shown that mesophyll cells in the wild-type leaf sheath adopt a C_3 differentiated state in that they accumulate the photosynthetic enzyme ribulose bisphosphate carboxylase (RuBPCase) (Langdale et al., 1988). In contrast, mesophyll cells of the wild-type leaf blade accumulate C_4-specific photosynthetic enzymes while RuBPCase accumulates specifically in bundle sheath cells (Fig. 3A). In situ localization of transcripts encoding the large subunit of RuBPCase (*rbcL*) demonstrate that in regions of *rs2* mutant leaf blades where veins are spaced more than four cells apart, *rbcL* transcripts accumulate in mesophyll cells (Fig. 3B). Similar patterns of accumulation are seen in regions of the leaf where ectopic midribs form i.e. *rbcL* transcripts accumulate in mesophyll cells (data not shown). Thus, in regions of *rs2* mutant leaf blades where vein spacing patterns are perturbed, mesophyll cells adopt a sheath fate.

Development of *rs2* leaves

In order to understand the origin of the phenotypes described above, a histological and SEM analysis of early leaf development was undertaken using 2-week old wild-type and *rs2* mutant plants. Wild-type maize leaf development is first characterized by the outgrowth of a leaf primordium on the periphery of the meristem flank and is referred to as the plastochron 1 stage (P1). This first point of outgrowth reflects the position of the future midvein and establishes the lateral axis of the leaf (Fig. 4A). Growth of the primordium out from the side of the meristem proceeds around the circumference of the shoot apex while the region corresponding to the future midvein lengthens rapidly to form a hood-like shape which covers the apex of the meristem at the P2 stage (Fig. 4A). The margins of the primordium meet and begin to overlap as rapid cell divisions throughout the primordium result in complete shrouding of the meristem and younger leaves (P3 stage) (Fig. 4B). At the P4-P5 stage, the leaf primordium expands in length and the margins continue to expand and wrap around each other forming a cone shape (Fig. 4C,D).

In *rs2* mutants, defects in leaf development are apparent immediately after initiation. Leaf primordia often have multiple lateral axes (typically 3; i.e. 3 midveins) and do not always encircle the circumference of the apex (Fig. 4E). At more advanced stages of development younger leaves can be observed growing over the edges of older primordia (Fig. 4F,G). This phenomenon is due in part to folding of the leaf margins toward the midrib. However, the primary cause may be that leaves are initiated too close to one another resulting in competition for founder cells and growth space such that a younger leaf grows over the marginal side of the next oldest leaf. For example, the P3 leaf shown in Fig. 4G forms a distinct boundary (marked by white arrow) where it is growing over the margin of the P4 leaf. This crowded form of

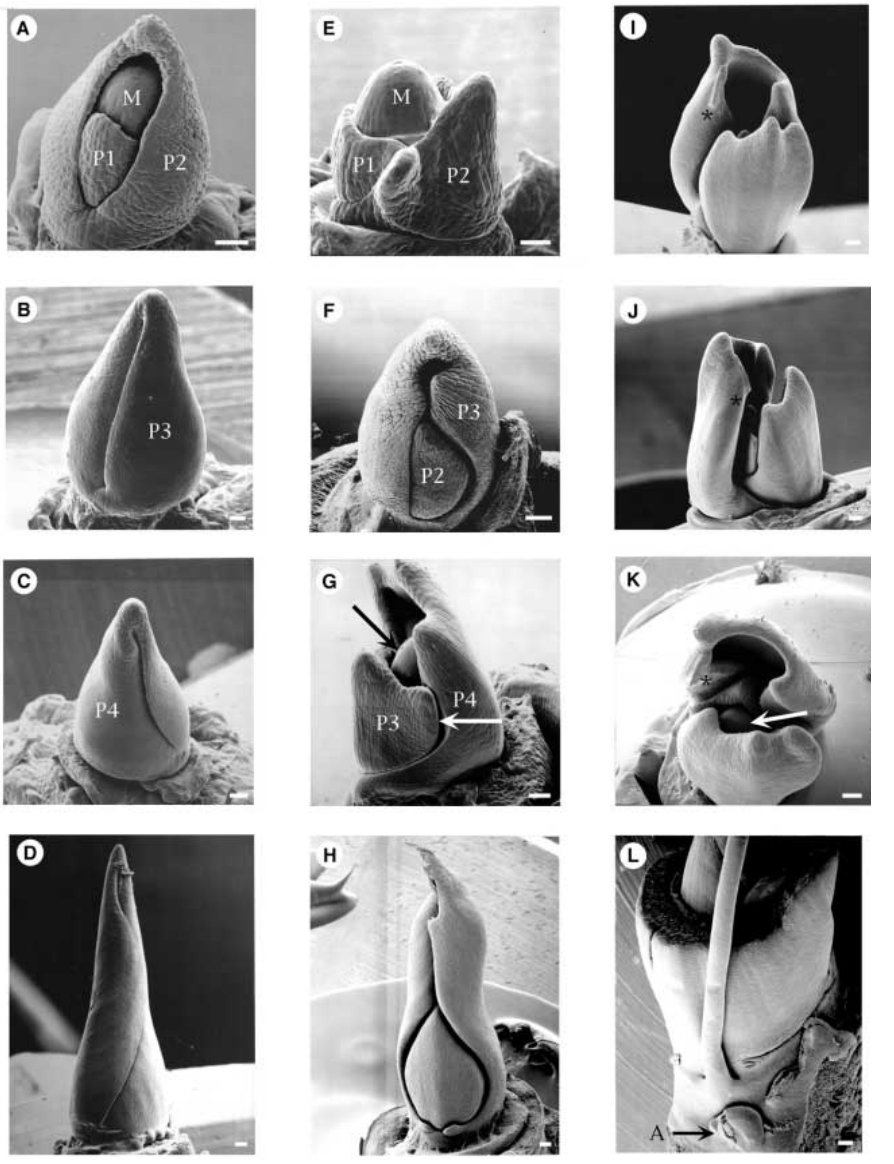

Fig. 4. SEM anlysis of leaf development in *rs2-R* mutants. *rs2-R* mutant leaf development is disturbed by the P1/P2 stage and is associated with incomplete insertion around the circumference of the shoot. (A-D) Wild-type apices; (A) apex showing meristem (M) and plastochron 1 (P1) and P2 leaf primordia. (B) P3 stage leaf primordium. (C) P4 stage leaf. (D) P5 stage leaf. (E-H) *rs2-R* mutant apices; (E) P1/P2 apex. (F) P3 apex showing underlying P2. (G) P3/P4 stage apex showing exposed meristem (black arrow) and abnormal leaf growth where the P3 leaf is growing over the margin of the P4 leaf (white arrow). (H) P4/ P5 stage apex. (I-K) Three views of a *rs2-R* apex showing P3, P4 and P5 leaves; Asterisk indicates reference leaf. Note that the margin of this leaf is severely folded in over its adaxial side. White arrow indicates meristem. (L) Example of a bladeless leaf inserted narrowly at the node; A, axillary bud. Size bars, 50 μm.

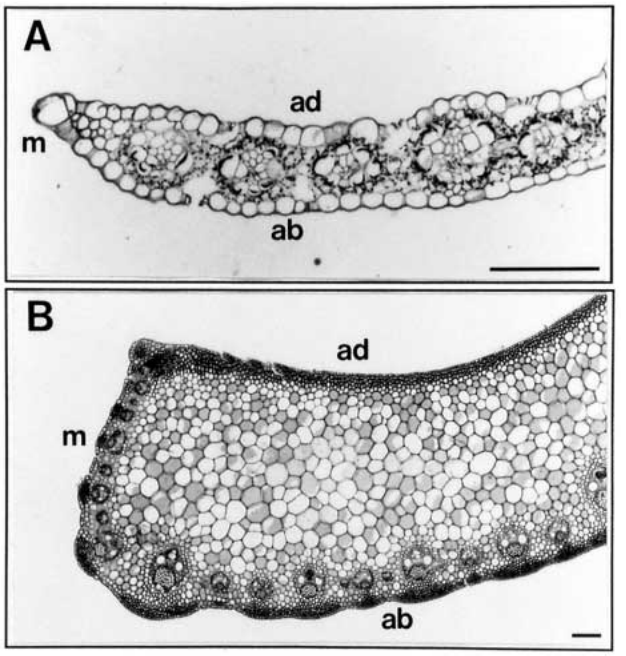

Fig. 5. Cellular arrangement in *rs2* bladeless leaves showing that dorsoventrality is maintained. Transverse section of the edge of mature wild-type (A) and bladeless *rs2-twd* mutant (B) leaves stained with Fast Green FCF. Ad, adaxial surface; ab, abaxial surface; m, margin. Size bar, 100 μm.

leaf development results in a restriction of leaf growth that becomes more pronounced with increasing leaf number (Fig. 4H,I,J,K). In some genetic backgrounds, leaf growth becomes so restricted that leaves resemble radially symmetric structures (Fig. 4L). However, transverse sections of fully developed leaves reveal that abaxial-adaxial symmetry is maintained in all cases (Fig. 5). *rs2* mutants also frequently develop wide leaves compared to wild type. The increase in width is accompanied both by the development of extra veins and by extra space between lateral veins (Fig. 3B and J. A. L., unpublished observations). Increased intervein space and widened leaves are also a regular feature of dominant *Kn1* mutants (Freeling and Hake, 1985).

Leaf initiation from axillary meristems is also irregular in *rs2* mutants. Fig. 6 shows an SEM comparison of prophyll and husk leaf initiation from wild-type and *rs2-R* axillary meristems. Whereas husk leaf initiation from the wild-type axillary meristem is distichous (Fig. 6A), a clear example of altered primordium initiation can be seen in the mutant apex (Fig. 6B). Here, two primordia of similar developmental stage are positioned on the same side of the meristem in an unusual phyllotactic arrangement. In addition, evidence of leaf margin folding similar to that seen in the primary apical meristem is visible (Fig. 6B).

Ectopic accumulation of KNOX proteins in *rs2* leaves

Since leaf development in maize is associated with a down-regulation of genes encoding KN1-like homeobox proteins, shoot apices of wild-type and mutant siblings were examined 2 weeks after germination using a KNOX antiserum that recognizes both KN1 and RS1 proteins and a RS1-specific antiserum (Scanlon et al., 1996). Fig. 7A and C show that in wild-type apices, KNOX proteins are present in most cells of the meristem. However, in the group of cells that constitute the incipient leaf primordium (P0), no protein is detected (Fig. 7A). Significantly, KNOX proteins are also absent from P0 cells in *rs2* mutant meristems (Fig. 7B) suggesting that the *rs2* gene may not play a role in the initial down-regulation of *knox* genes in the meristem.

As development proceeds in wild-type plants, KNOX proteins are down-regulated in cells as they become recruited into the primordium (Fig. 7C). In mutant meristems, which are shorter in height and more oval in morphology than wild-type, this recruitment process is less ordered. For example, $KNOX^-$ cells may extend only partially around one side of the apex creating an uneven ring with respect to normal phyllotaxy (Fig. 7D,G). The most striking phenotype observed in *rs2* mutant apices, however, is the ectopic accumulation of KNOX proteins in leaf primordia (Fig. 7B,D). As early as P1, KNOX proteins accumulate in the proximal regions of the primordium (Fig. 7B). Later in development, although a few leaves accumulate KNOX proteins throughout, ectopic accumulation is more frequently seen in 'patches' (Fig. 7D). This is observed both with the general KNOX antiserum and with the RS1-specific antibody (Fig. 7E). Particularly noticeable is the accumulation of RS1 protein in cells of the ligular region (Fig. 7F). These data are consistent with the idea that RS2 maintains wild-type cells of the leaf primordium in a KNOX-off state.

To investigate whether *rs2* regulates the expression of one or multiple homeodomain-encoding genes, we examined the expression of *kn1* and two other *knox* genes in wild-type and *rs2-R* immature sheath tissue. cDNA prepared from immature sheath tissue of leaf 7-8 (3 weeks after germination) was PCR amplified with primers specific for each of three *knox* genes. The predicted amplification products were 615 bp (*rs1*), 248 bp (*liguleless3; lg3*) and 342 bp (*kn1*). The results shown in Fig. 8 demonstrate that *rs1* and *lg3* transcripts are not present in wild-type leaves whereas *kn1* transcripts are detected at very low levels. In contrast, all three transcripts are present in *rs2* mutant leaves. Ectopic expression of *rs1* is particularly obvious. Ectopic expression was also observed when cDNA was prepared from mature leaf sheath tissue (data not shown). These results suggest that the *rs2* gene product acts as a general repressor of class I *knox* gene expression during leaf development.

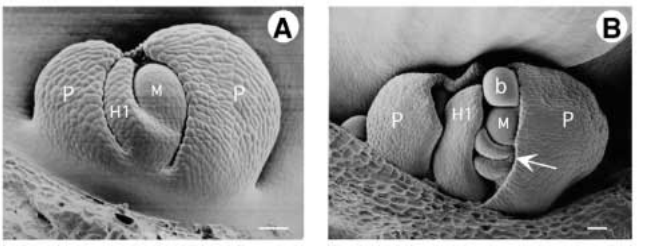

Fig. 6. Axillary bud development in normal and *rs2-R* mutant plants. Plants were harvested at 3-weeks old and meristems in the axils of leaf 3 examined. (A) Wild-type axillary meristem (M) exhibiting prophyll primordia (P) and a first husk leaf primordium (H1). (B) *rs2-R* axillary shoot showing the same organs as in A plus two small equally staged primordia in an abnormal phyllotactic arrangement; b, air bubble**.** Size bars, 50 μm.

DISCUSSION

Changes in regulatory gene expression patterns often mark a fundamental shift in the developmental potential of a cell. Although many studies have shown that leaf development is associated with down-regulation of *knox* homeobox genes, the mechanism by which this regulation is achieved is not understood. We have examined the role of the *rough sheath2* gene in leaf development and find that it defines a new class of genes that function to either directly or indirectly regulate plant *knotted1*-like homeobox genes. *rs2* mutants display a wide range of leaf phenotypes that resemble those caused by dominant mutations in several maize homeobox genes, such as *Kn1* and *Rs1* (Freeling and Hake 1985; Becraft and Freeling 1992). The most penetrant leaf phenotype is inappropriate cell fate acquisition whereby cells in the leaf blade adopt a sheath-like identity (Figs 1, 2, 3). Consistent with the phenotypic similarity to dominant homeobox mutants, *rs2* mutants show ectopic accumulation of KNOX homeodomain proteins in leaf primordia (Fig. 7). Transcript analysis further indicates that multiple *knox* genes including *kn1*, *rs1* and *lg3* are ectopically expressed (Fig. 8). We suggest that the *rs2* phenotype results from the elimination of a negative regulator, the *rs2* gene product, that normally represses *knox* gene expression in developing leaf primordia. Consistent with this model is the finding that the maize *rs2* gene, which has been cloned using the *rs2-twd* allele and *Spm* as a molecular tag, encodes a *myb*-like transcription factor (M. T., R. S., M. F. and J. A. L., unpublished observations).

In addition to the rough sheath phenotype, *rs2* mutant plants also show abnormalities in leaf shape, leaf insertion points and internode development. Thus it is probable that the *rs2* gene product also influences recruitment of cells into the leaf primordium. However, immunolocalization studies of even the most severe mutant plants indicate that KNOX proteins are down-regulated appropriately at P0 (Fig. 7). Thus, a mechanism that does not involve RS2 may be responsible for the initial down-regulation of KNOX proteins in the meristem. Due to the duplicated nature of the maize genome (Helentjaris et al., 1988), a second *rs2*-like gene may act to initially suppress KNOX accumulation at P0 while RS2 itself maintains cells in the leaf primordium in a KNOX-off state. However, transgenic plants expressing *knox* genes under the control of a constitutive promoter, also show appropriate repression of *knox* gene expression in PO (Chuck et al., 1996; Williams-Carrier et al., 1997). This would suggest that the initial down-regulation at P0 is facilitated by post-transcriptional mechanisms.

Two further aspects of the *rs2* phenotype, bladeless leaves and aberrant vascular patterning, provide an insight into mechanisms that may be operating during mutant leaf initiation and development. Bladeless and semi-bladeless leaves fail to encircle the stem, thus exposing the internode. Immunolocalizations of KNOX proteins during leaf initiation in *rs2* mutants suggest that this phenotype is correlated with a reduction in the number of cells recruited into the primordium at P1 (Fig. 7D,G). A similar observation in maize *ns* mutants led to the proposal that if founder cells fail to fully encircle the shoot apex then the leaf primodium lacks information for leaf margin development (Scanlon et al., 1996; Scanlon and Freeling, 1997). In support of this idea is the observation that,

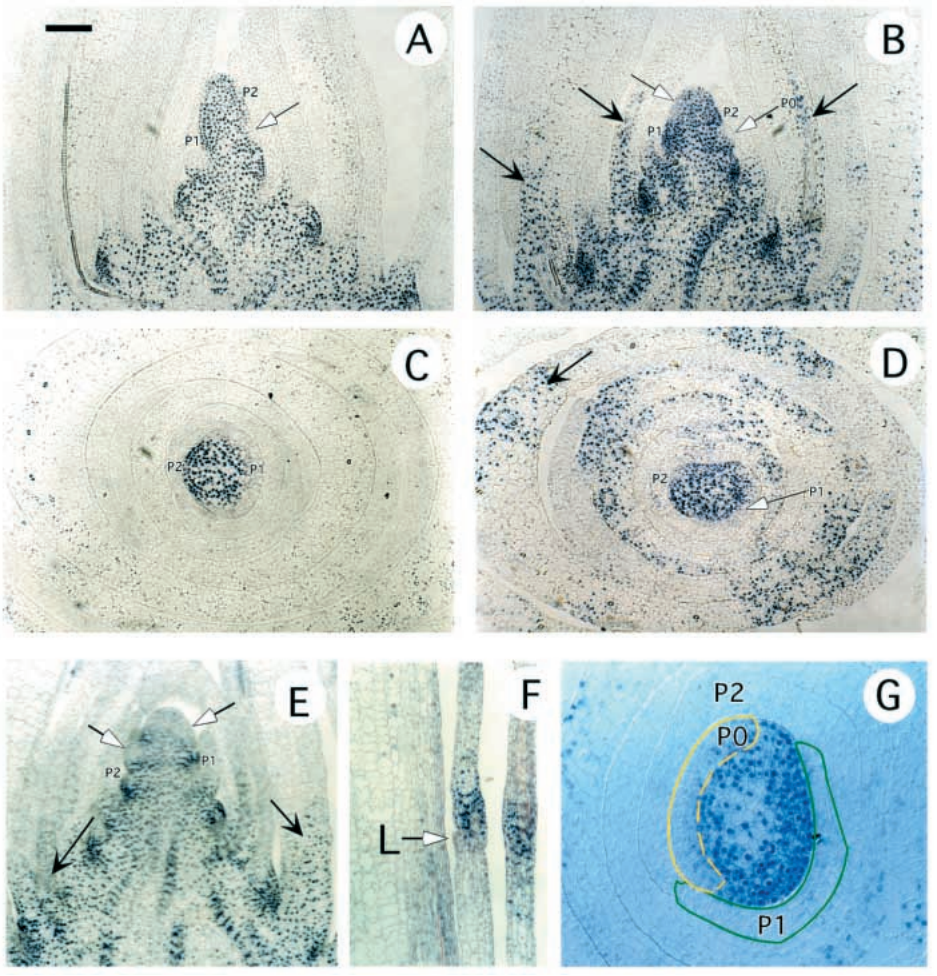

Fig. 7. KNOX proteins accumulate ectopically in *rs2* mutants. Immunolocalization of KNOX proteins in wild-type (A,C) and *rs2-R* (B,D,G) shoot apices. (E) Immunolocalization of RS1 protein in *rs2-R* vegetative shoot apex. (F) Ectopic accumulation of RS1 proteins in a P7 leaf at the site of ligule (L) development (white arrow). A,B,C and F are longitudinal sections whereas C, D and G are transverse sections. G shows a *rs2* apex with demarcation between P0 and P1 primordia. P0-P2 leaf primordia are indicated. Black arrows indicate ectopic accumulation of homeodomain proteins. White arrows in A,B,D and E indicate absence of KNOX proteins in P0 and P(-1) stage leaves. Size bar, 200 μm (A-E); 100 μm (F,G).

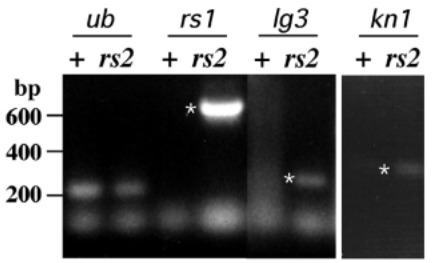

Fig. 8. *rs2-R* mutants ectopically express multiple *knox* genes. RT-PCR analysis of homeobox gene expression in wild-type (+) and *rs2-R* (*rs2)* immature leaves using gene-specific primers for *ubiquitin* (*ub*), *rough sheath1* (*rs1*), *liguleless3* (*lg3*) and *knotted1* (*kn1*). Asterisk indicates expected size of PCR products. The smear in the *lg3* lane represents non-specific amplification as determined by DNA gel blot analysis (data not shown).

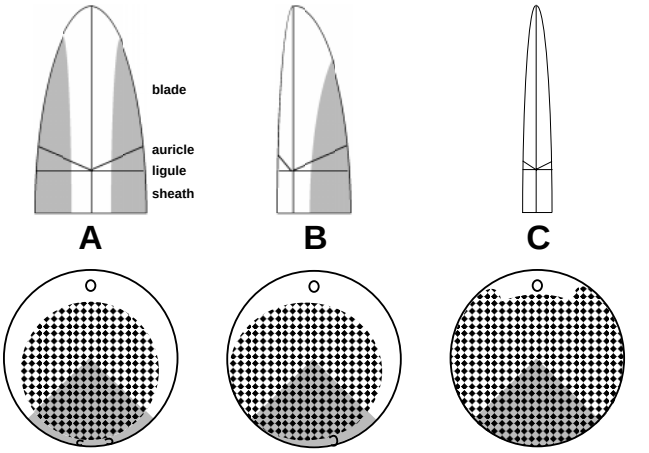

Fig. 9. Model for generating semi-bladeless and narrow leaves during leaf initiation in *rs2* shoot apices. The lower half of the figure represents transverse sections through the meristem as a P0-P1 stage leaf is formed. The upper half of the figure represents mature leaf phenotypes resulting from the meristematic configurations depicted. (A) Wild-type. Black and white diamonds indicate region of *knox* gene expression. White and grey regions peripheral to this *knox* region denote leaf founder cells. The grey region denotes the marginal domain defined by *ns* genes. (B,C) Model depicting how loss of *knox* gene down-regulation in a portion of the meristem leads to semi-bladeless and bladeless leaf phenotypes.

as seen in *ns* mutants, narrow leaves of *rs2* mutants have blunt edges and lack a tapering margin (Fig. 5B). *rs2;ns1;ns2* multiple mutant homozygotes appear additive in phenotype and mainly exhibit an increase in the narrowness of semi-bladeless leaves (R. S., unpublished observations). This suggests that NS and RS2 function in separate pathways and that NS function preceeds that of RS2. Failure to maintain down-regulation of *knox* genes in the marginal domain defined by the *ns* genes (Scanlon et al., 1996) could thus explain the elaboration of half leaf and narrow leaf phenotypes in *rs2* mutants (Fig. 9).

rs2 mutants exhibit significant aberrations in vascular patterning which range from an increased number of intermediate veins to the presence of multiple midribs. Since a large body of data suggest that auxin acts as an inductive signal for the development of vascular elements in the shoot (Sachs, 1991; Shininger, 1979), it seems reasonable to suggest that auxin homeostasis is perturbed in *rs2* mutants. Consistent with this idea is the fact that mutants that disrupt basipetal auxin transport, such as *lop1* in *Arabidopsis*, also exhibit oversized midribs and bifurcated midveins (Carland and McHale, 1996). Significantly, preliminary studies indicate that there are polar auxin transport defects in *rs2* mutants (M. T. and J. A. L., unpublished observations). Of interest with respect to these data are observations regarding maize homeobox gene expression and vascular differentiation. First, *kn1* and *rs1* homeobox gene expression is observed in vascular elements in the stem leading up to leaf traces (Smith et al., 1992; Jackson et al., 1994; Schneeberger et al., 1995). Second, ectopically expressed homeodomain proteins cause abnormalities in vein differentiation and may as a consequence disrupt the normal pattern of auxin transport (Becraft and Freeling, 1994; Sinha et al., 1993). Third, lateral vein formation in maize closely follows *knox* gene repression during leaf initiation (Smith et al., 1992; Schneeberger et al., 1995). Thus, the challenge now remains to determine the interelationship between developmental pathways that regulate *knox* gene expression and those that coordinate the action of plant growth regulators. However, one model for RS2 function that incorporates all of the above observations, is that the gene functions indirectly in early leaf development i.e. recruitment of cells into the primordium, and directly in late development i.e. repression of KNOX genes. This model would predict that *rs2* transcripts accumulate primarily after P1 and that signals from the developing primordia (possibly auxin?) influence the recruitment of cells from the apex into younger primordia.

Since *rs2* mutants exhibit floral phenotypes that are consistent with ectopic expression of *knox* genes in floral primordia (Jackson et al., 1994; Schneeberger et al. 1995; Kerstetter et al. 1997), it is likely that *rs2* negatively regulates *knox* gene expression during both vegetative and reproductive growth. Furthermore, since extra silks are produced from *rs2* mutant carpels (Fig. 1I,J,K) it is possible that *rs2* also regulates expression of the floral homeotic genes. As such, our results support the idea that genes like *rs2* are involved in specifying (or maintaining) a developmental state through the exclusion of genes which promote the elaboration of a different fate. A conceptually similar role has been proposed for the *CURLY LEAF* gene in *Arabidopsis* which acts to exclude *AGAMOUS* gene expression from leaves (Goodrich et al., 1997). It therefore becomes possible to view shoot development as the regulated expression of distinct developmental modules. Evolutionary variations in the temporal and spatial expression patterns of genes like *rs2* could thus explain the multitude of distinct shoot morphologies that are apparent in nature.

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