KNAT1 Induces Lobed Leaves with Ectopic Meristems When Overexpressed in Arabidopsis

George Chuck,a Cynthia Lincoln,b and Sarah Hakea, c,1

a Department of Plant Biology, University of California–Berkeley, Berkeley, California 94720
b Developmental, Cell, and Molecular Biology Group, Duke University, Durham, North Carolina 27708
c Plant Gene Expression Center, U.S. Department of Agriculture, 800 Buchanan Street, Albany, California 94710

Plant development depends on the activity of apical meristems, which are groups of indeterminate cells whose derivatives elaborate the organs of the mature plant. Studies of knotted1 (kn1) and related gene family members have determined potential roles for homeobox genes in the function of shoot meristems. The Arabidopsis kn1-like gene, KNAT1, is expressed in the shoot apical meristem and not in determinate organs. Here, we show that ectopic expression of KNAT1 in Arabidopsis transforms simple leaves into lobed leaves. The lobes initiate in the position of serrations yet have features of leaves, such as stipules, which form in the sinus, the region at the base of two lobes. Ectopic meristems also arise in the sinus region close to veins. Identity of the meristem, that is, vegetative or floral, depends on whether the meristem develops on a rosette or cauline leaf, respectively. Using in situ hybridization, we analyzed the expression of KNAT1 and another kn1-like homeobox gene, SHOOT MERISTEMLESS, in cauliflower mosaic virus 35S::KNAT1 transformants. KNAT1 expression is strong in vasculature, possibly explaining the proximity of the ectopic meristems to veins. After leaf cells have formed a layered meristem, SHOOT MERISTEMLESS expression begins in only a subset of these cells, demonstrating that KNAT1 is sufficient to induce meristems in the leaf. The shootlike features of the lobed leaves are consistent with the normal domain of KNAT1's expression and further suggest that kn1-related genes may have played a role in the evolution of leaf diversity.

INTRODUCTION

The tissues and organs of the adult plant originate from apical meristems, which are indeterminate, self-renewing groups of cells located at the root and shoot apices. Leaves initiate from the shoot apical meristem (SAM) as dorsiventral primordia (Hagemann, 1970; Kaplan, 1975). Whereas most leaves are determinate and differentiate from the tip down, the shoot, which includes the SAM, stem, and axillary buds, is indeterminate, is radially symmetrical, and differentiates from the base upward. One of the intriguing questions in plant development is how indeterminate cells of the SAM give rise to determinate primordia.

Studies of the knotted1 (kn1) homeobox gene of maize have shown that kn1 is expressed in the shoot portion of the plant. It is expressed strongly in the meristem and is downregulated before leaf initiation. Expression is also seen in the unexpanded stem and axillary buds (Smith et al., 1992; Jackson et al., 1994). The expression pattern in the meristem suggests that kn1 may play a role in maintaining indeterminacy in the meristem and/or repressing differentiation. kn1 overexpression in tobacco results in minuscule leaves with ectopic shoots on the leaf surface (Sinha et al., 1993), consistent with the idea that kn1 functions in the meristem.

To extend our knowledge of homeobox gene function into dicotyledonous plants, related genes were cloned from Arabidopsis by using the kn1 gene from maize. Two of these genes, designated KNAT1 and KNAT2, encode proteins with, respectively, 53 and 40% amino acid identity compared with KN1 overall, and 89 and 80% identity within the homeodomain (Lincoln et al., 1994). A third gene, SHOOT MERISTEMLESS (STM), is also very similar to kn1, with 47% predicted amino acid identity overall and 92% identity within the homeodomain (Long et al., 1996). STM is the first kn1-like gene described to have a loss-of-function phenotype (Long et al., 1996). In stm mutants, embryogenesis occurs through the cotyledon stage, but the remaining components of the shoot system are never made. Cells that occupy the position of the SAM in the torpedo-stage embryo have the characteristics of mature differentiated cells rather than small, densely staining cells typical of the meristem (Barton and Poethig, 1993). This finding implies that STM is needed to repress differentiation and to keep the cells of the SAM in an indeterminate state.

KNAT1 is expressed in the meristem and stem (Lincoln et al., 1994) in a way similar to the expression of many kn1-like homeobox genes of maize (Jackson et al., 1994). No transcript
has been detected in leaves or roots. Downregulation of KNAT7 occurs in the peripheral zone as leaves initiate, which is similar to the results found for knl. In the inflorescence stem, expression is restricted to the cortex cells adjacent to the vascular strands leading up to the base of early flower primordia. Expression has not been detected in any determinate lateral organs, roots, or flowers, except for a small group of cells in the style (Lincoln et al., 1994).

Overexpression of KNAT7 under the control of the cauliflower mosaic virus 35S promoter revealed a dramatic lobed-leaf phenotype in two transformants (Lincoln et al., 1994). Here, we extend our studies to include additional transformants and show that a subset of these have ectopic SAMs in the sinus region at the base between two lobes. The phenotype suggests that KNAT7 has endowed the leaf with characteristics of a shoot. We discuss the implications of these findings in the broader context of the knl gene family.

RESULTS

Generation and Analysis of Transformants

We used vacuum infiltration (Bechtold et al., 1993) to generate 29 independent Arabidopsis lines carrying the KNAT7 cDNA driven by the cauliflower mosaic virus 35S promoter (Jefferson et al., 1987). All transformants were confirmed to be independent and to have the 35S::KNAT7 transgene, as shown by DNA gel blot analysis, and resistance to kanamycin in the T2 progeny (data not shown). Twenty-one of the 29 primary (T0) transformed lines, including one isolated previously by Lincoln et al. (1994), displayed a lobed-leaf phenotype in the T1 progeny to varying degrees. The transformants were placed in four phenotypic categories based on leaf morphology: (1) normal phenotype, (2) mild phenotype with large leaves and slight lobes or prominent serrations, (3) moderate phenotype with small leaves and large lobes, and (4) severe phenotype with small, deeply lobed leaves, as shown in Table 1. Figure 1A compares a wild-type Nossen leaf with the mild, moderate, and severe 35S::KNAT7 phenotypes. In general, the severity of the lobing phenotype correlated with a smaller lamina and wider petiole. The flowers of the severe class were smaller and bore thin, elongated, greenish petals that abscised early and anthers that dehisced later than normal (Figure 1B). Flowers of the mildy lobed and normal classes appeared normal. Time to flowering and phyllostaxy were unaffected in all lobed transformants, except for plants in the severe class that grew more slowly and took longer to flower under long-day conditions.

To determine whether RNA levels correlated with phenotypic severity, we isolated RNA from leaves of individual T2 kanamycin-resistant plants from 14 independent transformants chosen to represent the different categories. The resulting RNA blot was probed with the KNAT7 cDNA. Figure 2 shows that 35S::KNAT7 RNA levels did not correlate with phenotypic severity. Although some normal transformants had low to non-detectable amounts of RNA, other transformants in the moderate class had even lower levels of RNA. Transformants in the severe class had high levels of RNA, although one transformant in the mild class had even higher levels. A similar lack of correlation between phenotypic severity and overall expression levels has been found in tomato (Hareven et al., 1996) and mildly lobed tobacco transformants overexpressing the maize knl gene (Sinha et al., 1993) as well as in tobacco plants overexpressing the maize ZmHox1a or ZmHox1b genes (Überlacker et al., 1996). The results suggest that factors other

<table>
<thead>
<tr>
<th>Leaf Phenotype</th>
<th>Transformant (No.)</th>
<th>Ectopic Floral Meristems</th>
<th>Ectopic Vegetative Meristems</th>
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<tr>
<td>Severely lobed</td>
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a Severely lobed transformants have very small, thick, dark-green leaves with large lobes and deep sinuses. Rosette diameter ranges from 0.5 to 3.0 cm.
b (+), presence; (−), absence.
c Moderately lobed transformants have small leaves and large lobes. Rosette diameter ranges from 4.0 to 6.0 cm.
d Mildly lobed transformants have large leaves and slight lobes with very shallow sinuses. Rosette diameter is near that of the wild type.
than the overall amount of KNAT1 transcript detected in mature leaves regulate the degree of lobing.

**Histological Analysis of Lobed Leaves**

Sections of lobed leaves were compared with wild-type leaves to determine the effect of ectopic KNAT1 expression on leaf cell differentiation, as shown in Figure 3. Wild-type leaves have an organized internal anatomy containing a layer of vertically packed palisade cells at the adaxial or upper surface. Spongy parenchyma cells, interspersed with air spaces, are loosely arranged below the palisade layer (Figures 3A and 3C; Pyke et al., 1991). Severely lobed 35S::KNAT1 leaves do not contain a recognizable palisade parenchyma layer. In addition, there is at least one extra layer of small, tightly packed mesophyll cells with greatly reduced air spaces (Figures 3B to 3F). Changes in both the pattern and number of vascular elements were also seen in all 35S::KNAT1 transformants with lobed leaves. Wild-type leaves have a continuous closed

![Figure 1. Phenotypes of KNAT1 Transformants.](image)

(A) Rosette leaves of wild-type Nossen and individuals representative of the mild, moderate, and severe categories of the KNAT1 transformants, from left to right, respectively. Small, lobed leaves are characteristic of the moderate and severe categories of the KNAT1 transformants.
(B) Flower of a severely lobed KNAT1 transformant (left) and wild-type Nossen (right).
(C) Close-up of vegetative leaf of a severely lobed transformant with ectopic shoot meristems in the sinus region.
(D) Cleared fourth rosette leaf tip of the wild type showing xylem elements.
(E) Cleared fourth rosette leaf tip of a severely lobed transformant with altered veins.
(F) Cleared cauline leaf of a severely lobed transformant showing an ectopic inflorescence with flowers and siliques. The vasculature of the inflorescence originates from a single vein of the lower sinus (arrowhead).
venation pattern evident at the leaf tip. Connections to other veins, such as the midvein, are established at this point (Figure 1D). In contrast, veins of 35S::KNAT1 lobed leaves rarely connect and instead abruptly at the tip of the leaf (Figure 1E). The midvein of the severely lobed transformants has an abnormally large number of vascular elements, and all higher order veins have approximately twice the number of xylem elements as does the wild type (Figure 1E). Localized cell expansion normally associated with wild-type midveins was also evident around first- and second-order branched veins leading to lobes in 35S::KNAT1 plants (Figure 3D).

Lobes Initiate in the Position of Serrations

We used scanning electron microscopy to determine at what point in development the morphology of 35S::KNAT1 leaves deviated from that of the wild type. Soon after a wild-type leaf initiates from the SAM, a pair of stipules develops from the outer margin cells located at its base, as shown in Figure 4A (Medford et al., 1992). As the leaf matures, serrations form along its margin in a basipetal direction (Figure 4B). The first two leaves usually lack serrations, and the number of serrations increases on leaves that are formed later (Telfer and Poethig, 1994). Early stages of 35S::KNAT1 leaf development are identical to those of wild-type leaves in all respects, although the 35S::KNAT1 leaves are smaller (compare Figures 4B and 4D). The morphology of lobed leaves diverges from that of the wild type after serrations are initiated, at which point the serrations become prominent dorsiventral primordia (Figure 4E) that develop into lobes. As the lobe forms, one or two pairs of stipules initiate on each side from cells at the outermost margin of the lamina. One to four ectopic stipules can be found in each sinus (Figures 4C and 4F), and occasionally they line the apical portion of the petiole as well. Using trichomes as markers for leaf maturation (Figure 4B), we observed the lobes develop and differentiate in a basipetal direction similar to the entire leaf (Figure 4F). Often, second-order lobes appeared on lobes initiated after the first pair (Figure 4F). In general, a pair of lobes first becomes apparent on the third leaf, and the lobes then progressively increase in number on later-formed leaves such that the fourth and fifth leaves have two and three pairs of lobes, respectively. Thus, lobes occupy the position of serrations but resemble leaf primordia by having ectopic stipules at their bases.

Meristems Initiate in the Sinuses of the Lobed Leaves

Late in the development of six independent transformants (Table 1), ectopic inflorescence meristems form on cauline leaves in the sinus of the most basal pair of lobes. If the branch in the axil of the cauline leaf is removed, these ectopic meristems continue to grow and eventually form morphologically normal, fully functional flowers with viable seed. Clearing of cauline leaves showed that the entire vasculature of the ectopic inflorescence originated from a single vein of the cauline leaf leading to the sinus (Figure 1F).

We also observed four independent transformants that formed ectopic vegetative meristems in the sinuses of the rosette leaves late in development (Figure 1C and Table 1). Unlike the inflorescence meristems that develop singly in the basal sinuses, the vegetative meristems appear in groups in all sinuses. Some of the rosette-born meristems switch to the floral phase late in development, after the main apex has begun to senesce.

Sections through sinuses during the early stages of both vegetative and floral ectopic meristem formation revealed that these meristems always form directly opposite the xylem pole of a well-developed vein (Figures 3E and 3F). The ectopic meristems show a continuity of layers with surrounding leaf cells, suggesting that they did not arise endogenously. Cells within the meristem are smaller and more densely cytoplasmic than the progressively larger cells seen surrounding the meristem. The dome of the early inflorescence meristem displays a three-layered tunica corpus-type organization, which is similar to that of the wild type (Figure 3E).

A normal pattern of cell differentiation occurs within the tissues and organs derived from the ectopic meristems, despite their abnormal position. Cuboidal vacuolated pit cells central to the procambium are present, similar to cells in the normal stem (Figure 3F). The abaxial and adaxial surfaces of leaf primordia differ in the extent of their vacuolation early in their development, similar to wild-type leaves (Hagemann, 1970). In addition, as lateral organs initiate, procambial strands from leaf primordia connect to the preexisting vein (Figure 3F).

Both floral and vegetative ectopic meristems arise from a unique subset of cells that is located on the adaxial leaf surface and is not precisely at the margin where ectopic stipules form, as shown in Figure 5. Ectopic vegetative meristems were

![Figure 2. RNA Gel Blot Analysis of 35S::KNAT1 Transgenic Lines.](image-url)
Figure 3. Histological Analysis of Leaves from the Wild Type and Severely Lobed 35S::KNAT1 Transformants.

(A) Transverse section of a wild-type rosette leaf, including the midvein. Bar = 100 μm.

(B) Transverse section of a 35S::KNAT1 rosette leaf, including the midvein and enlarged secondary veins. Bar = 100 μm.

(C) Close-up of a transverse section from the wild type showing palisade and spongy parenchyma. Bar = 50 μm.

(D) Close-up of a transverse section from a 35S::KNAT1 leaf showing an abnormal vein, tightly packed cells, and lack of palisade parenchyma. Bar = 50 μm.

(E) Transverse section of a cauline leaf from a 35S::KNAT1 transformant with an initiating inflorescence meristem over a vein. Bar = 25 μm.

(F) Transverse section of a 35S::KNAT1 rosette leaf with an initiating vegetative meristem over a vein. A procambial strand is differentiating between the leaf primordia and the existing vein. Bar = 25 mm.

The arrows indicate the positions of a vein within each leaf. mv, midvein; pc, procambial strand; pp, palisade parenchyma; sp, spongy parenchyma.
Figure 4. Scanning Electron Microscopy of Immature Leaves.

(A) Initiating wild-type rosette leaf with emerging stipules. Bar = 7 μm.
(B) Wild-type fourth rosette leaf with serrations (se) and basipetally differentiating trichomes. Bar = 88 μm.
(C) Close-up of a sinus of a severely lobed 35S::KNAT1 transformant with four ectopic stipules, which form from the outermost margin. Bar = 29 μm.
(D) Fourth rosette leaf of a severely lobed 35S::KNAT1 transformant with two pairs of serrations. Bar = 34 μm.
(E) Initiating ectopic stipules at the base of a future lobe. Bar = 14 μm.
(F) Older fourth rosette leaf of a severely lobed 35S::KNAT1 transformant with basipetally differentiating lobes. The lower right lobe has initiated secondary lobes. Stipules can be seen at the sinus. Bar = 168 μm.

Arrowheads indicate stipules in (A), (C), (E), and (F).
first detected as radially symmetrical groups of cells. Subsequently, one or more leaf primordia became visible (Figure 5A), with additional leaves initiating directly opposite the previous leaf (Figure 5C). Usually, vegetative meristems are found in clusters or rows. In rare cases, the meristems spread into the lamina, leaving a trail of abnormal shoots with dozens of leaf primordia (Figure 5G). These rows of meristems always follow preexisting veins, as revealed by cleared leaves (data not shown). Younger leaf primordia sometimes initiate between older primordia or at the ends of the elongated meristem (Figure 5G) but never in a spiral phyllotaxy. Leaves formed from the ectopic meristems are similar to wild-type vegetative leaves in that they have stipules at their bases and trichomes differentiating from their adaxial tips, but they differ in that they sometimes display partial fusions with adjacent leaf primordia (Figure 5E).

Ectopic inflorescence meristems were also detected as radially symmetrical groups of cells on the adaxial leaf surface, but lateral organs or floral meristems were not produced immediately. In contrast to the development of ectopic vegetative meristems, a period of stem elongation occurs during which the inflorescence meristem is elevated above the surface of the leaf (Figure 5B). One or more cauline leaves containing additional inflorescence meristems in their axils initiate during the elongation phase, followed by the production of a few floral meristems in a helical pattern (Figure 5D). The pattern of lateral organ differentiation from ectopic floral meristems appears to be identical to that of the wild type (Figure 5F; Smyth et al., 1990).

Expression of KNAT1

We performed in situ hybridization by using a KNAT1 antisense probe to determine whether variation in the transgene expression pattern could explain the location of the ectopic meristems. As shown in Figure 6B, 35S::KNAT1 was strongly expressed around the developing veins of young leaves and at lower levels in older leaves. Preferential KNAT1 expression, however, was not detected in the sinus regions before meristem formation (data not shown). Thus, the high levels of KNAT1 expression in the veins may explain why meristems initiate in the vicinity of veins but fail to explain why the sinus is targeted for meristem formation instead of other regions of the leaf.

In situ hybridization experiments were also performed to analyze the development of ectopic meristems. In ectopic vegetative meristems that were just initiating, KNAT1 expression was seen in a small group of cells in all three cell layers overlaying the vein (Figure 6A). After several periclinal cell divisions, the meristem became evident as a bulge of cells on the surface of the leaf (Figure 6B, lower leaf). Areas lacking KNAT1 expression in the ectopic meristems are likely to be sites of leaf initiation (Figures 6A and 6B), as previously demonstrated for KNAT1 expression in SAMs (Lincoln et al., 1994). 35S::KNAT1 expression returned in older leaf primordia as procambial strands differentiated (data not shown).

**STM Is Expressed after Meristems Form**

STM is expressed in the SAM early in embryo development (Long et al., 1996). To determine when adaxial leaf cells express STM during the development of ectopic meristems, in situ hybridization was performed using an STM antisense probe. A meristem at a stage equivalent to the one shown in Figure 6A did not display STM expression (data not shown). Expression of STM was first detected in a small group of cells after the ectopic meristem had established a three-layered tunicate corpus and had initiated periclinal divisions (Figure 6C). After additional growth, STM expression expanded to include all underlying cell layers of the meristem (Figure 6D). Expression was not found in the outer epidermal layer, or L1, which was similar to the results found for kn1 RNA localization in maize (Jackson et al., 1994) but dissimilar to findings for STM expression in wild-type SAMs. No STM expression was evident around the veins or throughout the sinus region. As seen for KNAT1 and STM in the SAM (Lincoln et al., 1994; Long et al., 1996), STM expression disappeared as leaves initiated and eventually was restricted to a flattened strip of cells located at the apex of the meristem (Figure 6E).

**DISCUSSION**

Here, we describe the effects of KNAT1 overexpression on Arabidopsis leaf development. Like kn1 in maize and STM in Arabidopsis, KNAT1 is expressed in the vegetative meristem and stem and is downregulated as leaf primordia are initiated. By overexpressing KNAT1 ectopically, we changed the simple Arabidopsis leaf into a lobed leaf with stipules in the sinus of the lobes. In a subset of the transformants, ectopic meristems formed in the sinus region in close vicinity to veins. Identity of the ectopic meristem depended on whether the leaf was floral or vegetative. The shootlike characteristics of these leaves are consistent with the normal domain of KNAT1 expression and provide implications for the role of kn1-like genes in the evolution of leaf diversity.

**Lobes as Extended Serrations**

Like the leaves of most plants, Arabidopsis leaves display subtle heteroblastic development (Telfer and Poethig, 1994) in which early formed leaves are morphologically different from later ones. The first two foliage leaves of Arabidopsis are small and spoon-shaped with smooth, rounded edges. From the third leaf on, a pair of serrations forms along the margin at the mid-point of the lamina. Primary veins branching from the midvein tend to terminate in each serration. Later leaves are larger than the first two and become progressively more serrated and elongated but are never lobed (Telfer and Poethig, 1994). We showed that KNAT1 overexpression conditions a lobed-leaf phenotype in Arabidopsis. In most transformants, lobes
Figure 5. Scanning Electron Microscopy of Initiating Meristems at the Sinus of Severely Lobed 35S::KNAT7 Transformants.
Induction of Meristems by KNAT1

first appeared on the third leaf and then gradually increased in number until the floral stage. This gradual increase in lobbing is reminiscent of the initiation and progression of serrations in wild-type leaves. Veins terminated in the tips of lobes in a fashion similar to the termination of veins in wild-type serrations. In addition, scanning electron microscopy of 35S::KNAT1 leaf primordia showed that the lobes developed from what began as serrations. Thus, the timing and position of lobes in transgenic plants suggest that the lobes are extensions of serrations rather than abnormal outgrowths.

The transformation of serrations to dorsiventral leaflike primordia could result from an increase in growth of the serration or a lack of growth in the sinus region. Analysis of leaf development in other species has demonstrated that differential growth rates contribute dramatically to shape changes. For example, *Tropaeolum majus* has round leaves, whereas *T. peltophorum* has acutely lobed leaves. It has been shown that although the leaf primordia appear similar in these species, the sinus region grows faster relative to the lobe region in *T. majus* (Whaley and Whaley, 1942), whereas in *T. peltophorum*, the lobes and sinuses maintain a similar growth rate. Differences between tomato leaf shape mutants have also been attributed to growth-rate changes (Dengler, 1984). We suspect that the lobes result from a lack of growth in the sinus due to the overall small size of the 3SS::KNAT1 leaves.

**Position and Identity of Ectopic Meristems**

Meristems form in a subset of the transformants at the base of the lobes on the adaxial surface. The meristems are always closely associated with the vasculature. Two models could explain why meristems form near veins. The first model suggests that KNAT1 alters hormone levels that in turn cause veins to initiate and ectopic shoots to form. Auxin is thought to be a major regulatory factor in vasculature differentiation, and various cytokinins promote xylem differentiation (Aloni, 1987). In addition, tobacco plants transformed with the Agrobacterium isopentenyl transferase gene, which leads to the synthesis of cytokinin, also produce ectopic shoots from veins (Schmülling et al., 1993, 1998; Li et al., 1992).

The second model takes into account the fact that the 3SS promoter is strongly expressed near veins (Jefferson et al., 1987; Schneider et al., 1990). In situ hybridization with the KNAT1 antisense probe revealed high levels of expression specifically around the veins (Figure 6B). Only background expression was detected in other leaf tissues at the time when ectopic meristems were initiating. Thus, ectopic meristems may form near veins simply because KNAT1 expression is highest in that area. These experiments do not resolve the contribution of endogenous KNAT1 gene expression to the ectopic meristem, nor do they determine whether the reduced expression outside of veins results from degradation of 35S::KNAT1 RNA.

Whether meristems form near veins due to KNAT1 expression levels or due to hormone concentrations still does not explain the position of meristems in the sinus. Tissue sectioning through sinuses before the onset of meristem initiation showed that cells occupying the sinus are generally smaller and have cell walls that stain differently from the rest of the leaf (data not shown). This histological observation could reflect a fundamental difference in the competence of sinus cells to respond to developmental signals. Leaf margins in general remain densely cytoplasmic for a longer period of time than do cells in the center of the leaf and are the last to differentiate (Hagemann and Gleissberg, 1996). In addition, the lobes differentiate basipetally, and thus, the base of the lobe differentiates after the tip of the lobe. The combined influences of the margin and the lobes would make the sinus region the last to differentiate. Troll (1939) first noted that ectopic leaf shoots form in the part of the leaf that is last to mature. The less differentiated cells within the sinus may be more competent to respond to KNAT1 (or some secondarily derived signal) and organize to form a meristem.

We envision a two-stage process in the development of ectopic meristems in the sinus region. In the first stage, KNAT1 expression causes a lobed-leaf phenotype in which the sinus region matures last. In the second stage, cells within the sinus respond to signals emanating from developing higher order veins by initiating ectopic meristems. These signals could be KNAT1 itself or secondary signals such as hormones. A two-stage process in which meristems develop due to conditions set up in the sinus may explain the lack of strong KNAT1 expression in the sinus itself.

Ectopic meristem initiation in tobacco transformants over-expressing kn1 also occurs directly over leaf veins but at the center of the leaf near the petiole (Sinha et al., 1993). In addition, tobacco leaves that bear shoots are small and not lobed.

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**Figure 5.** (continued).

(A) Initiating ectopic vegetative meristem with three leaf primordia.
(B) Initiating ectopic inflorescence meristem with a cauline leaf primordium.
(C) Row of ectopic vegetative meristems with leaf primordia that emerge opposite to one another.
(D) Ectopic inflorescence meristem with initiating floral meristems.
(E) Cluster of ectopic vegetative meristems. The leaf primordia have stipules at their bases. Some of the leaf primordia appear to be fused.
(F) Ectopic inflorescence with cauline leaves and maturing floral buds.
(G) Rows of ectopic vegetative meristems emanating from the sinus.

cl, cauline leaf; im, inflorescence meristem; lp, leaf primordia. Arrowheads in (C), (E), and (F) indicate stipules. Bars in (A) to (D) and (F) = 30 μm; bars in (E) and (G) = 50 μm.
Figure 6. In Situ Localization of KNAT1 and STM RNA in Developing Ectopic Vegetative Meristems of Severely Lobed Transformants by Using Antisense Probes.

(A) KNAT1 expression in small dividing cells that are presumed to form an ectopic meristem. Note the proximity to a vein.
(B) KNAT1 expression in the vasculature in the younger leaf (upper) and expression in ectopic vegetative meristems in the older leaf (lower). Ip, leaf primordia.
(C) STM expression in an initiating ectopic vegetative meristem.
(D) STM expression in an older ectopic vegetative meristem.
(E) STM expression in an older ectopic vegetative meristem with developing leaves.

The arrows in (A) to (C) and (E) indicate a vein. Bar in (A) = 10 μm; bars in (B) to (E) = 50 μm.

These differences probably reflect differences in how tobacco and Arabidopsis leaves develop; when we analyzed the phenotype of KNAT1 and kn1 in both Arabidopsis and tobacco, we found that the phenotype depends on the species, not on the construct. The fact that the original Kn1 mutation of maize also results in ectopic leaf vein expression (Smith et al., 1992), but with different developmental consequences, again argues that unique plant factors play a role in the gain-of-function phenotypes for kn1 and the kn1-like homeobox genes.

Cauline leaves produce inflorescence meristems in their sinuses, whereas rosette leaves produce vegetative meristems, even when the plant is flowering. This finding suggests that the ectopic KNAT1 gene product is capable of interacting with endogenous factors present in the leaf to induce meristems with specific identities. One such factor present in cauline leaves may be the product of the LEAFY gene, which is necessary for floral meristem identity. The LEAFY transcript is found in wild-type cauline leaves (Weigel et al., 1992) and in the basal margins of cauline leaves on 35S::KNAT1 plants (G. Chuck, unpublished data). The ability of 35S::KNAT1 to condition ectopic inflorescence and floral meristems is interesting in light of the fact that KNAT1 expression is absent from those meristems (Lincoln et al., 1994). This result suggests that the KNAT1 gene product may play an indirect role in flowering by
activating or interacting with other genes, such as LEAFY, which are necessary for floral meristem identity.

Meristems Initiate before STM Expression

We expected that ectopic meristem formation would closely correlate with expression of STM. stm mutants fail to progress beyond the production of cotyledons (Barton and Poethig, 1993). Expression of STM is seen first in the late globular embryo before cotyledon formation in a band of cells between the presumptive cotyledons. Expression persists in that region as cotyledons expand (Long et al., 1996). Our in situ analysis showed that STM expression does not occur until after leaf cells have formed an ectopic meristem, indicating that STM expression is not strictly required to initiate a meristem. This finding is consistent with models suggesting that STM may not be required to maintain the meristem after it has initiated (Hake et al., 1995). Although it is difficult to surmise function from gain-of-function phenotypes, the expression of KNAT7 in the meristem and its ability to ectopically produce meristems suggest that KNAT7, like STM, will be required for meristem initiation or maintenance.

Apical-Basal Transformations in kn1 Gain-of-Function Phenotypes

Class 1 members of the kn1 gene family (Kerstetter et al., 1994) are expressed in the shoot portion of the plant, that is, the SAM, axillary buds, and stem, and are restricted from expression in the leaf (Jackson et al., 1994; Lincoln et al., 1994; Ma et al., 1994; Müller et al., 1995). Dominant mutant phenotypes caused by ectopic expression of kn1-like genes in the leaf (Smith et al., 1992; Müller et al., 1995; Schneeberger et al., 1995) can all be explained as a shift of basal characteristics into apical regions within the framework of the phytomer, the basic repeating unit of the plant. Each phytomer contains a leaf, an axillary bud, and an internode (the stem segment between leaves) (Gray, 1879). The floral shoot of monocots can also be envisioned as a series of compact phytomers (Galinat, 1959; Bossinger et al., 1992). In some dominant mutants, the shift is a histological transformation within the leaf, from the base of the leaf toward the tip of the leaf. In other cases, the shift is from the stem or axillary bud into the leaf. For example, leaves of Kn1 mutants are characterized by the presence of sheath and auricle tissues, which are normally positioned lower than the blade, in the blade portion of the leaf (Freeling and Hake, 1985; Sinha and Hake, 1994). In Rough sheath1 mutants, the auricle region resembles the sheath in epidermal histology but also has overall characteristics of the stem (Becraft and Freeling, 1994; Schneeberger et al., 1995). This apical shift of basal leaf identities also occurs in 3SS::KNAT7 transformants, as determined by the presence of ectopic stipules between the lobes. The severely lobed transformants with ectopic meristems could be interpreted as representing a more dramatic shift in which basal components, such as axillary buds, are displaced apically into the blade. Axillary buds normally form at the base of the leaf (Hempel and Feldman, 1994). A similar transformation is seen in the dominant Hooded mutation of barley, in which floral meristems arise on the awn, a leaflike structure of the flower, due to ectopic expression of the kn1 ortholog in barley (Müller et al., 1995). A combination of competency based on intrinsic factors in each plant's development and the timing of gene expression is a likely explanation for the variations in the dominant phenotypes.

Role of kn1 Genes in the Evolution of Leaf Morphology

Certain characteristics of leaflet initiation on dissected leaves resemble the initiation of leaf primordia from the SAM (Lacroix and Sattler, 1994). Sattler has argued that dissected leaves evolved from simple ones through the process of homeosis, that is, dissected leaves may have arisen by the imposition of shootlike properties onto a simple leaf program. The kn1 class of homeobox genes could be important players in such a process. We have shown that ectopic expression of KNAT7 in the leaf confers shootlike properties on the leaf, such as the ability to initiate leaflike lobes as well as ectopic meristems. Moreover, ectopic expression of other homeobox genes may condition different subsets of the KNAT7 overexpression phenotype because 3SS::KNAT2-transformed Arabidopsis plants also have the lobed-leaf phenotype, albeit without ectopic stipules or meristems (G. Chuck, unpublished data).

Ectopic expression of kn1-like homeobox genes in leaves of other species could have played a role in the evolution of leaf morphology. The kn1 homolog in tomato, which has dissected leaves, is expressed in leaf primordia, unlike all other class 1 kn1-like genes examined thus far (Hareven et al., 1996). Furthermore, ectopic expression of the maize kn1 gene in tomato conditions many additional orders of leaf dissection (Hareven et al., 1996). It will be interesting to determine whether kn1-like homeobox genes are expressed in leaf primordia in other plants with dissected leaves. The results presented here suggest that these genes can serve as a starting point for molecular dissection of the variation in leaf shape and development in other species.

METHODS

Plant Growth Conditions

Arabidopsis thaliana (ecotype Nossen) seeds were grown under a 16-hr light and 8-hr dark cycle at 22°C. For vacuum infiltration, 10 to 20 seeds were sown in 5.5-inch pots over soil covered with nylon mesh. Ten pots were used regularly per infiltration experiment. For in situ hybridization experiments, leaves were harvested from 4-week-old bolting plants grown under long-day conditions (Lincoln et al., 1994) and fixed in 4% paraformaldehyde (Jackson, 1991). For analysis of
kanamycin segregation, progeny of primary transformants were germinated on in vitro germination media supplemented with 50 mg/mL kanamycin under a 16-hr photoperiod at 22°C.

Arabidopsis Transformation

A construct containing the cauliflower mosaic virus 3SS promoter driving the KNAT7 cDNA in the binary vector pBIN19 (Lincoln et al., 1994) was transformed into Agrobacterium tumefaciens GV3101 by electroporation. Vacuum infiltration into Arabidopsis was performed as described, with only minor modifications (Bechtold et al., 1993). Thirty-nine primary transformants (T₀) were recovered and transplanted to soil after 1 week. Twenty-nine of these segregated kanamycin-resistant progeny in the next generation (T₁) after self-pollination. T₁ progeny were transferred to soil after 1 week and scored for morphological phenotypes. The presence of the transgene in T₂ lines was confirmed by DNA gel blotting.

DNA and RNA Gel Blot Analyses

RNA was isolated from rosette leaves and blotted according to the method of Smith et al. (1992). DNA was isolated from pooled rosette leaves of transgenic plants by using the method of Chen and Dellaporta (1994). DNA gel blots were performed, as described by Lincoln et al. (1994), by using 5 μg of DNA digested with SstI and probing with a 1.0-kb SstI-BamHI fragment from the 3' end of the KNAT7 cDNA. Hybridizing bands not found in the wild-type line Nossen were counted as single T-DNA insertions.

In Situ Hybridization

Antisense transcripts for KNAT7 subclones were synthesized with T3 polymerase by using nucleotides labeled with digoxigenin-11-UTP (Boehringer Mannheim). A 1.4-kb EcoRI subclone of the KNAT7 cDNA lacking the 3' end but containing the homeobox was used for all in situ hybridizations. This fragment gave expression patterns identical to those of a probe that did not contain the homeobox (Lincoln et al., 1994). Antisense transcripts were made to a region of SHOOT MERISTEMLESS (STM) spanning amino acids 81 to 382, including the homeodomain (Long et al., 1996). Although they are closely related by sequence, we are confident that KNAT7 does not cross-hybridize to STM, because a near full-length KNAT7 in situ probe does not reveal expression in floral and inflorescence meristems, as shown for STM (Long et al., 1996). Cross-hybridization to other knotted1 (kn1)-like homeobox genes in leaves is unlikely because expression of class 1 kn1-like homeobox genes is restricted from leaves (Jackson et al., 1994; Kerstetter et al., 1994). Probe hydrolysis, hybridization, and washes were done as described by Jackson (1991). Sections were passed through an ethanol series and Histoclear (National Diagnostics, Atlanta, GA) before mounting in Merkoglas (EM Science, Cherry Hill, NJ).

Whole-Mount Leaf Preparations

Mature leaves were fixed in 7:1 ethanol to acetic acid overnight and then cleared with a chloral hydrate-glycerol-water solution (8:1:2 [w/v]). Dark-field photographs were taken with Kodak Royal Gold 100 print film by using an Axioskop (Carl Zeiss, Inc., Thornwood, NY).

Light Microscopy

Leaves of various stages were fixed in FAA (3.7% formaldehyde, 50% ethanol, 5% acetic acid, 45% water) under vacuum for 10 min on ice, dehydrated in an ethanol series to 95%, and embedded in JB-4 embedding medium (Polysciences, Inc., Warrington, PA). Sections were cut at 2 μm with tungsten knives on a rotary microtome HM 340 (Carl Zeiss), mounted on slides, and stained with periodic acid–Schiff's reagent and 0.1% toluidine blue.

Scanning Electron Microscopy

Leaves were fixed in either FAA or 4% glutaraldehyde overnight at 4°C and then dehydrated in an ethanol series to 100% the next day. Samples were then critical point dried, mounted on aluminum stubs, and viewed on a scanning electron microscope at 10 kV.

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