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Phil. Trans. R. Soc. Lond. B 1995 **350**, 45-51
doi: 10.1098/rstb.1995.0136

Homeobox genes in the functioning of plant meristems

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SUMMARY

The maize homeobox gene *knotted1* (*kn1*) is expressed in vegetative and floral meristems and is down-regulated at the site of primordia formation. *kn1*-related genes from maize and other species also show meristem-specific expression and offer additional tools for studying the activities of shoot meristems. Members of this gene family are expressed early in embryogenesis, providing molecular markers for meristem initiation. Ectopic expression of either *kn1* or a related *Arabidopsis* gene, *KNAT1*, causes dramatic alterations in *Arabidopsis* and tobacco leaf morphology. Most significantly, meristems form on the leaf, producing small shoots. We discuss whether the phenotypes can be interpreted as changes in positional information or timing of determination.

1. INTRODUCTION

Plants offer a special advantage to the study of primordia formation in that they generate large numbers of organs sequentially during development. The primordia are also arranged in specific patterns allowing precise predictions to be made as to the site of future primordia.

The ability to continually generate organs resides in the meristem, stem cell-like populations that maintain themselves while also producing determinate and indeterminate organs. Histological analysis of shoot apical meristems shows that cells in the central zone divide less frequently and are more vacuolated than those in the periphery. Primordia arise from the peripheral zone. Meristems can give rise to two types of primordia: determinate primordia such as leaves or petals, or indeterminate primordia such as floral meristems. Meristems also produce the cells that contribute to the growth of the stem. In woody plants,

	helix I	helix II turn helix III	
class1			
KN1	SKKKKKGKLPKEARQQLLSWWDQHYKWPYPSETQKVALAESTGLDLKQINNWFINQRKRHWKPS		maize
OSH1	..........D.....N..EL.........S..............................		rice
RS1	..............K..H..EL..........E.I.........Q..................		maize
KNOX4	..............K..H..EL..........E.I....A....Q..................		maize
KNAT1	..............K..T..EL.........SE..........Q..................		arabidopsis
KNOX8	..R...........K..H..EL..........E.M....T....P.................A		maize
KNOX3	C..R..D........K.....EL..R......ME.I.........EQ..................		maize
SBH1	M..R.............E..NR.........S..L.........Q..................		soybean
KNAT2	..........R....A..D..NV.N.....T.GD.IS...E....Q..................		arabidopsis
KNOX10	.R.........RD...K..H..QL..R.......LE.A.........EA..............QA		maize
LG3	L..R.......D..TV..E..NT..R....T.ED..R..AM....P..................		maize
KNOX5	L..R.......D..SA.MD..NT..R....T.ED..R..AM....P..................		maize
KNOX11	L..R.......D..SA.MD..NT..R....T.ED..R..AA....P..................		maize
class2			
KNOX1	LR.RRA....GDTTSI.KQ..QE.S.....T.DD.AK.V.E...Q.............N.HNN		maize
KNOX2	LR.RRA....GDTAST.KA..QA.S.....T.ED.AR.VQE...Q.............N.HNN		maize
KNOX6	MR.RRA....GDTASV.KA..QA.S.....T.DD.AR.VQE...Q.............N.HSN		maize
KNOX7	MR.RRA....GDTASV.KA..QA.S.....T.DD.AR.VQE...Q.............N.HSN		maize
BNHD1	MR.RRA....GDTTTV.KN..Q..C.....T.DD.AK.V.E...Q.............N.HNN		*Brassica napus*
other			
ATH1	QIWRPQRG..EKSVSV.RN.MF.NFLH...KDSE.HL..IRS..TRS.VS.....A.V.L...M		arabidopsis

Figure 1. Amino acid sequence comparisons of *kn1*-like genes. The sequences are divided into two major classes based on sequence similarity and expression patterns (Kerstetter *et al.* 1994).

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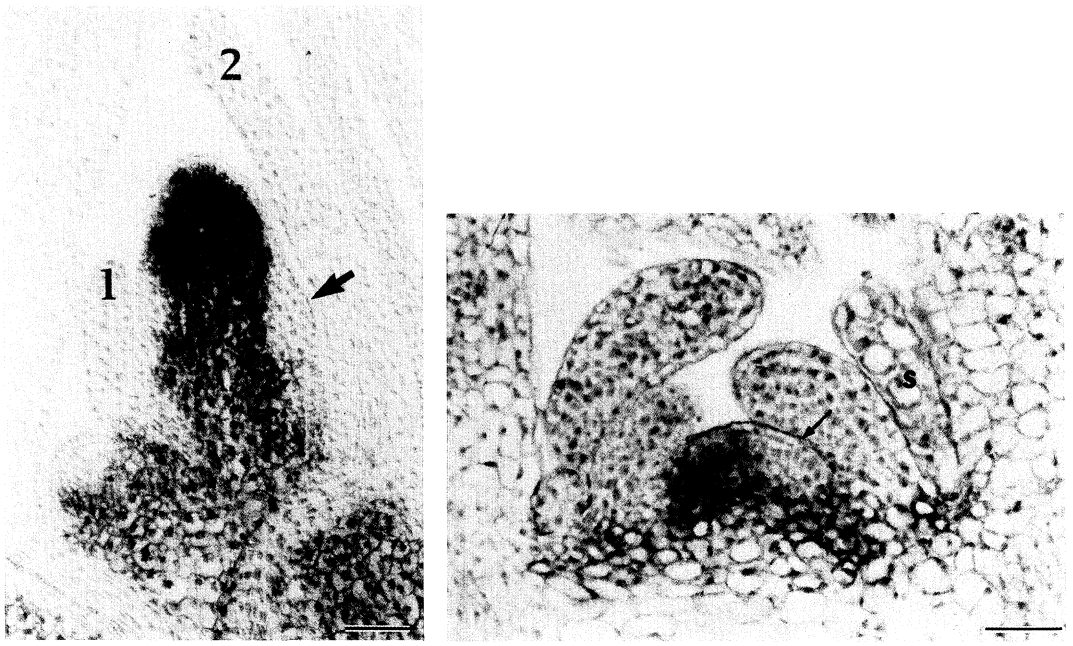

Figure 2. *In situ* hybridizations of *kn1*-like genes in the vegetative meristems. *kn1* (left panel) and *knat1* (right panel) are expressed in the vegetative meristems of maize and *Arabidopsis*, respectively (Jackson *et al.* 1994; Lincoln *et al.* 1994). Expression disappears before primordia formation.

the stem contains a distinct population of meristem cells – called the vascular cambium – that contributes to the increase in girth of the plant. The features of a meristem invite a number of questions: (i) how does a meristem form; (ii) how does a meristem produce determinate structures; and (iii) how is a determinate primordium distinguished from an indeterminate primordium? To answer these questions, a gene whose product marks the meristem would be extremely useful. In this paper, we show how the expression patterns and gain of function phenotypes of *kn1* and related gene family members suggest these genes are appropriate markers for the meristem, and allow us to address the questions raised.

2. *kn1* GENE FAMILY

knotted1 was cloned by transposon tagging a dominant leaf mutation (Hake *et al.* 1989). Sequencing of the cDNA showed that it encodes a homeodomain, a well characterized DNA-binding domain (Vollbrecht *et al.* 1991). The *kn1* homeobox was used as a hybridization probe to isolate a number of related genes from maize, called *knox* for *kn*otted like homeo*box*, (Kerstetter *et al.* 1994) as well as genes from other species (Matsuoko *et al.* 1993; Boivin *et al.* 1994; Lincoln *et al.* 1994; Ma *et al.* 1994; Quaedvlieg *et al.* 1995). The genes isolated fall into two classes by sequence comparisons; class 1 genes are 73–89% identical to *kn1* in the homeodomain, whereas class 2 genes are 55–58% identical to *kn1* in the homeodomain (see figure 1).

Expression studies have shown that class 1 genes are expressed in the meristem and stem, but are excluded from determinate organs such as leaves (Jackson *et al.* 1994; Kerstetter *et al.* 1994; Lincoln *et al.* 1994). The disappearance of the *kn1* gene product occurs before leaf initiation (see figure 2). In maize, *kn1* is down-regulated in a crescent-shaped ring of cells around the meristem (Smith *et al.* 1992; Jackson *et al.* 1994), the thickest portion of which corresponds to the region of

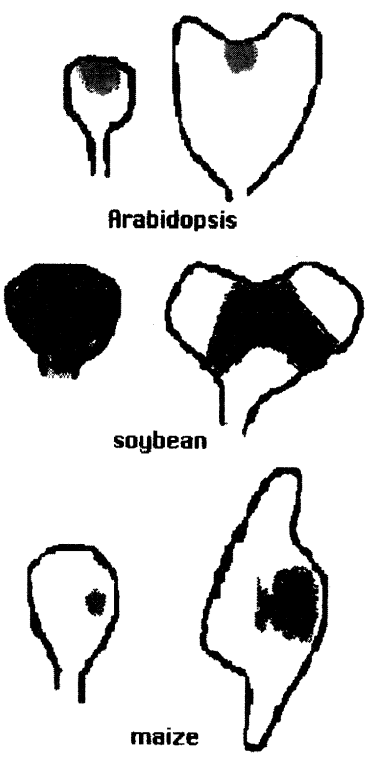

Figure 3. Schematic drawing of the expression patterns of *kn1*-like genes in *Arabidopsis*, soybean and maize embryos.

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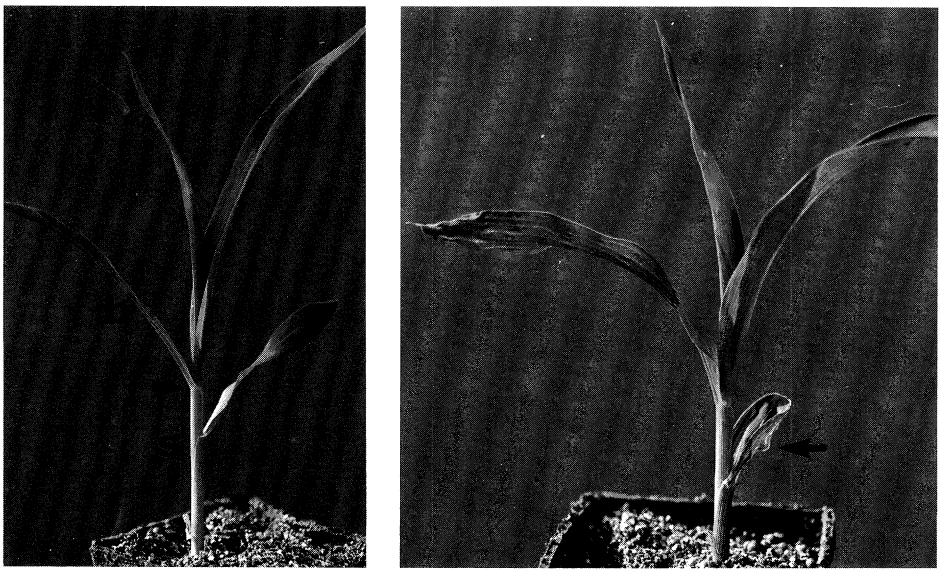

Figure 4. Normal and knotted seedlings. The position of the ligule is marked with an arrowhead. The blade is above the ligule and the sheath below it. The knots at the tip of the leaf are indicated with an arrow.

the future midrib. In *Arabidopsis*, which has a much smaller meristem, the region where *KNAT1* gene expression (*knotted1*-like in *Arabidopsis thaliana*) disappears is relatively larger, including almost a third of the meristem (Lincoln *et al.* 1994). By examining transverse serial sections, the absence of *KNAT1* expression appears to predict the spiral leaf initiation pattern of *Arabidopsis* (C. Lincoln, unpublished data).

3. INITIATION OF THE MERISTEM

The shoot apical meristem and the cotyledons (first leaves of the embryo) form during embryogenesis. In maize, a number of leaves are also initiated. Exactly when the shoot apical meristem forms and what it encompasses has been a matter of debate (Kaplan 1969; Goldberg *et al.* 1994). Because a meristem is defined in part by the organs it makes, it is difficult to determine the presence and extent of the meristem before organ initiation. Comparative morphological analysis suggests that the meristem initially comprises the apical half of the globular embryo and produces the cotyledons from the peripheral zones, similar to the manner in which leaves are produced (Kaplan 1969; D. Kaplan & T. Cooke, personal communication). Mutant analysis in *Arabidopsis* has led to a different interpretation of when the meristem forms. The *shoot meristemless* (*stm*) mutant only makes cotyledons at the shoot pole. One possible interpretation of this mutant is that the gene product is required to make a shoot meristem, suggesting that the cotyledons do not form from the meristem (Barton & Poethig 1993). An alternative interpretation, however, is that the gene product is needed for the meristem to renew itself such that in the *stm* plants, cotyledon initiation depletes the meristem.

Expression of *kn1* and related genes during this early stage of development has been examined in soybean, *Arabidopsis*, and maize. In soybean, *SBH1* is expressed throughout the globular embryo and disappears in the lateral regions as the cotyledons expand (Ma 1994) (see figure 3). Expression of an *Arabidopsis kn1*-like gene is confined to a central stripe within the apical half of the globular embryo (J. Long, personal communication). If *kn1*-like genes mark the meristem, then in these two dicot examples, the shoot apical meristem

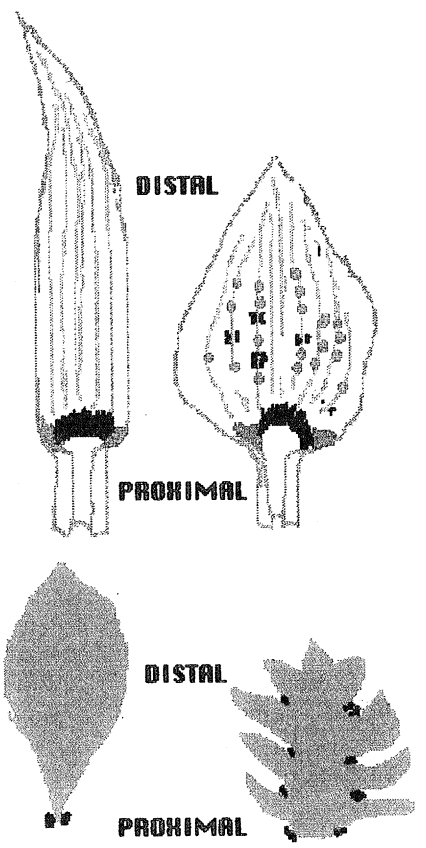

Figure 5. Positional model for *kn1* and *knat1* gain of function phenotypes.

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forms early in the globular embryo stage prior to elaboration of the cotyledons.

Maize, a monocotyledonous plant, has a single cotyledon called the scutellum. The early divisions of the embryo are irregular and concentrated in the apical region, producing a club-shaped proembryo. The first histological signs of the shoot apical meristem begin in a lateral position on the embryo a few days after pollination. The divisions become more regular and an epidermal layer forms (Randolph 1936). The domain of *kn1* expression corresponds to the meristem as defined by Randolph (see figure 3). Expression begins in a subset of the embryo cells and expands to include a larger domain. Growth of the coleoptile, the ensheathing base of the scutellum, and elaboration of leaves delimits the zone of expression (Smith *et al.* 1995). *kn1* expression may initially encompass the cells that form the coleoptile but does not seem to include all the cells of the scutellum. Does this suggest that the maize shoot meristem does not initiate the scutellum? Perhaps, but other possibilities remain such as the presence of *kn1* expression at very low levels through out the apical half of the maize proembryo (D. Jackson, unpublished data), or the possibility that an additional, unidentified *knox* gene is expressed earlier in embryogenesis. It is clear, however, that expansion of the cotyledons and/or embryonic leaves directly follows the disappearance of *kn1* in all three species examined, and may require the down-regulation of *kn1* in those cells.

4. GAIN OF FUNCTION PHENOTYPES

The *kn1* locus was first defined by a series of dominant mutations that alter development of the maize leaf (Gelinas *et al.* 1969; Freeling & Hake, 1985). A normal maize leaf contains three major regions, the blade, the ligule-auricle region, and the sheath (see figure 4). The ligule is an epidermal fringe at the junction between the blade and sheath, while auricles are wedge-shaped regions adjacent to the ligule. Each region of the leaf has characteristic cell types. *Kn1* mutant leaves contain distinct alterations to cells along the vasculature of the blade. 'Knots' result when foci of cells continue to divide and elongate. Because these cells are constrained by the adjacent regions, they grow out of the plane of the leaf in a focal point. Cells along the veins that do not develop into knots have sheath identity as determined by epidermal and internal cellular characteristics (Sinha & Hake 1994). In fact, the knots themselves can be considered sheath-like due to the basipetal (tip to base) differentiation of maize leaves. At a certain time in leaf development, the blade will be fully differentiated but the sheath will be still growing (Sharman 1942; Sylvester *et al.* 1990). Therefore, cells within the knotted blade that divide inappropriately into a knot may be responding to sheath-determining signals.

Normal leaves do not express *kn1* at any time in their development, however, in mutant leaves there is expression of *kn1* in cells that will differentiate to become vein cells (Smith *et al.* 1992). Expression is first detected when the leaf is in the fifth plastochron, i.e. the fifth leaf counting from the meristem. These cells are committed to being leaf cells, but are not yet fully differentiated into particular cell types. It is clear that the ectopic expression leads to the knotted phenotype, however, we have not been able to explain why the presence and severity of knots is sporadic despite the fact that the ectopic expression of *kn1* appears uniform along the veins.

The dominant leaf mutation *Rough Sheath1* (Becraft & Freeling 1994) also corresponds to a *knox* gene (Schneeberger *et al.* 1995). The ligule/auricle region of the leaf is affected in *Rs1* plants, whereas the blade and sheath are fairly normal. We have preliminary data to suggest that an additional dominant leaf mutation, *Gnarley1*, corresponds to another *knox* gene, *knox4*. The *knox4* gene and the *Gn1* mutant phenotype are tightly linked on chromosome 2L. More importantly, *knox4* is ectopically expressed in *Gn1* leaves, suggesting that *knox4* is, in fact, *Gn1*. Although it is possible that *knox4* is induced in *Gn1* leaves but does not actually correspond to *Gn1*, we have not yet found an example where one *knox* gene induces another to be expressed in ectopic positions (Jackson *et al.* 1994, and unpublished data). *Gn1* plants are most affected in the sheath region, but the stem itself is also disturbed.

The dominant maize leaf phenotypes suggest that *kn1* and other *knox* genes control cell fate determination, but do not necessarily imply a role for these genes in the meristem itself. The restriction of the mutant phenotype to a change in leaf cell fate may result from temporal and spatial limitations on the ectopic expression of *kn1*. To examine the effect of constitutive expression, we transformed *Arabidopsis* and tobacco with *kn1*, *KNAT1* and *knox3* cDNAs driven by the cauliflower-mosaic-virus promoter, 35S. Analysis of 35S:kn1 tobacco plants revealed a correlation between the amount of KN1 present and the severity of phenotype. Low levels of KN1 did not give a phenotype, plants with moderate amounts of KN1 had a lobed leaf phenotype, and high levels of KN1 produced short bushy plants with extremely small leaves. Most strikingly, shoots formed on the small leaves (Sinha *et al.* 1993). We have seen similar phenotypes with 35S:knox3 and 35S:KNAT1, but have not analysed the phenotypes for quantitative differences.

Transgenic *Arabidopsis* plants overexpressing either *kn1* or *KNAT1* have highly lobed leaves. The lobing is first observed in the third leaf and includes cauline leaves (Lincoln *et al.* 1994). Unlike the diminutive tobacco leaves, these lobed leaves are not significantly smaller, although they are shorter and wider than wild-type. At present, only two 35S:KNAT1 transformants have been analysed in detail. In one transformant, the regions between the lobes, the sinuses, have remarkable morphological features. The sinuses of most vegetative leaves contain 2–3 stipules. Stipules are normally found only at the base of the leaf where it attaches to the stem and first appear as the leaf primordium begins to expand (Medford *et al.* 1992). Also, flowers or flowering branches form in the sinuses

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of cauline leaves in addition to the stipules. This same line has produced vegetative shoots in the sinuses of rosette leaves following an EMS mutagenesis experiment to isolate enhancers and suppressors.

5. DISCUSSION

At least three possible interpretations can be made of the *kn1* or *KNAT1* gain-of-function phenotypes. One possibility centres on the interpretation of position by the cells with altered fates, another focuses on the timing of determination, and the third (argued by Freeling and colleagues with regard to the maize phenotypes only) focuses on a putative leaf maturation schedule (Freeling 1992). The first two interpretations are discussed here because the third has been well argued. *kn1* mutants are characterized by cells in the blade which have sheath fates. Because the blade is always more distal than the sheath, we can think of this alteration as a change in positional information whereby leaf blade cells respond to sheath signals. Occasionally, sheath tissue is also affected by large knots that encompass many veins. Because leaves grow and differentiate in a distal to proximal wave, any spurious growth in more distal regions, including knots on the upper regions of sheaths, could be considered the adoption of proximal fates. The other dominant mutations such as *Rs1* or *Lg3* also show displaced sheath into blade tissue (Freeling 1992), although the *Gn1* phenotype may include some blade into sheath alterations. A proximal/distal misidentity also explains the ectopic stipules seen in the *Arabidopsis KNAT1* transformants since they are normally found only at the base of the leaf (see figure 5). How do we explain the presence of ectopic shoots on leaves? We could start by asking what is the proximal boundary to the leaf. Is it the place where the leaf attaches to the stem, or the stem itself? In many plants, it is difficult to draw a distinct line between leaf and stem, especially in plants where the lower leaf zone is clearly part of the stem. Additionally, leaf and stem can be regarded merely as specializations of the shoot (Foster & Gifford 1974). Perhaps the most proximal position is the shoot apical meristem itself, in which case the presence of meristems in leaves is the most dramatic identity change possible.

The other interpretation focuses on the concept of determination. Meristems are indeterminate in contrast to leaves which are determinate. Because the tip of a leaf differentiates before the base, there exists a time period in maize leaf development when the sheath is less determined than the blade. Therefore, when cells in the blade have sheath identity, they could be interpreted as having adopted a leaf fate determined later in development. When leaf cells become meristems, they adopt a fully indeterminate state.

Is the leaf homologous to a shoot in the presence of constitutive *kn1* expression? The tobacco leaves on flowering plants are more radially symmetrical (Sinha *et al.* 1993). Each lobe of the 35S:KNAT1 *Arabidopsis* leaves could be considered a leaf because stipules form at the bases of these lobes. The flowers that form in the sinuses between the lobes could be interpreted as flowering shoots in the axils of the leaf lobes. However, the lobed leaves are not radially symmetrical nor do they differentiate from the base upward as is typical of a shoot. Therefore it seems more appropriate to argue that cells within the leaf have become meristem-like rather than arguing that the leaf has been transformed into a shoot.

We have shown that *kn1* is expressed in the shoot apical meristem from its inception. It continues to be expressed in shoot meristems and disappears only as determinate organs are initiated. *kn1* expression disappears from floral meristems as determinate lateral organs form and disappears all together when the inner whorl of organs, the carpels, is initiated. *kn1* expression in leaves alters leaf morphology, but most strikingly, causes the production of shoots on the leaves. In light of these observations, it seems reasonable to suggest that this gene is an appropriate marker for shoot meristem activity. Given this supposition we will address the questions raised in the introduction.

1. How does a meristem form? We imagine that initiation of the embryonic meristem requires a number of genes including *kn1*-related genes. In maize, there are hundreds of embryo lethal mutations in which the endosperm forms but development of the embryo itself is blocked at early stages (Clark & Sheridan 1991). Some of these genes may encode products needed to make the shoot apical meristem. The *defective kernel shootless Mu8* (*dks8*) mutant may be such a mutation: in severe mutants, a normal scutellum and root pole forms, but the shoot apical meristem, leaves and coleoptile do not form (C. Rivin, Oregon State University, personal communication). This phenotype adds support to the idea that a scutellum can form in the absence of a shoot apical meristem, consistent with the expression patterns of *kn1*. An embryo mutation in *Arabidopsis*, *raspberry*, blocks development at the globular embryo stage. Cotyledons and leaves fail to form although cell differentiation proceeds normally (Yadegari *et al.* 1994). The primary defect of *raspberry* could be lack of cotyledon formation which then blocks further progression of primordia initiation. It would be interesting to determine what the *kn1* expression patterns are in these mutants, perhaps *kn1* is on, but never turned off in the incipient organ positions.

The presence of ectopic meristems in transgenic plants that overexpress *kn1* or related genes suggests that *kn1* is sufficient for ectopic meristem formation. We suspect that *kn1* acts by tapping into existing plant developmental processes. For example, *kn1* may affect the ratio of plant growth regulators such as auxin to cytokinin. Overexpression of a cytokinin biosynthetic gene also leads to the production of shoots on leaves (Estruch *et al.* 1991; Li *et al.* 1992). In addition, the senescence of *kn1* transgenic tobacco leaves is greatly delayed similar to plants that have been treated with exogenous cytokinin or plants that are ectopically expressing the cytokinin producing gene (Smart *et al.* 1991; reviewed by Brzobohatý *et al.* 1994). It is also possible that ectopic *kn1* expression mimics cytokinin overproduction because *kn1* is downstream of cytokinin. Another possible way by which *kn1* may tap into normal developmental mechanisms is through vein

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initiation; some *kn1* transgenic plants have a great number of veins. Whether the extra veins cause a change in hormone levels, or hormone changes result in altered venation is unknown.

2. How does the meristem produce determinate organs? Our evidence indicates that *kn1* mRNA and protein disappear at the site where determinate primordia are made, therefore, removal of the *kn1* gene product may be a prerequisite for producing a determinate organ.

3. How is a determinate primordium distinguished from an indeterminate primordium? To answer this question, we have examined expression of *kn1*-like genes during the initiation of floral meristems in three different species. A *kn1*-related *Arabidopsis* gene is expressed in the inflorescence meristem and disappears on the flanks of the inflorescence prior to floral meristem formation. Gene expression returns as the meristem primordia expand (J. Long, unpublished data). In contrast, expression of *kn1* in the maize inflorescence appears to persist through the stages of spikelet initiation into floral meristem formation (Smith *et al.* 1992). Unlike either *Arabidopsis* or maize, a sunflower *kn1*-like gene is not expressed in the flat capitulum although it is expressed earlier in the vegetative shoot apical meristem. Expression of this *kn1*-like gene returns to predict the position of floral meristems (D. Jackson, unpublished data). Thus all three genes are expressed in floral meristems but differ in the transition to a floral meristem; expression begins before primordia initiation in sunflower, expression continues without interruption in maize, and expression disappears before initiation of the floral primordium in *Arabidopsis* but reappears in the primordium. Regardless of these differences, we can distinguish a determinate primordium from an indeterminate one by the presence of *kn1* in the latter.

We wish to thank D. Kaplan and K. Barton for discussions, and C. Rivin for allowing us to mention her *dks8* mutant. Thanks to R. Wells for manuscript preparation. The work was supported by grants from the NSF and USDA to S.H.

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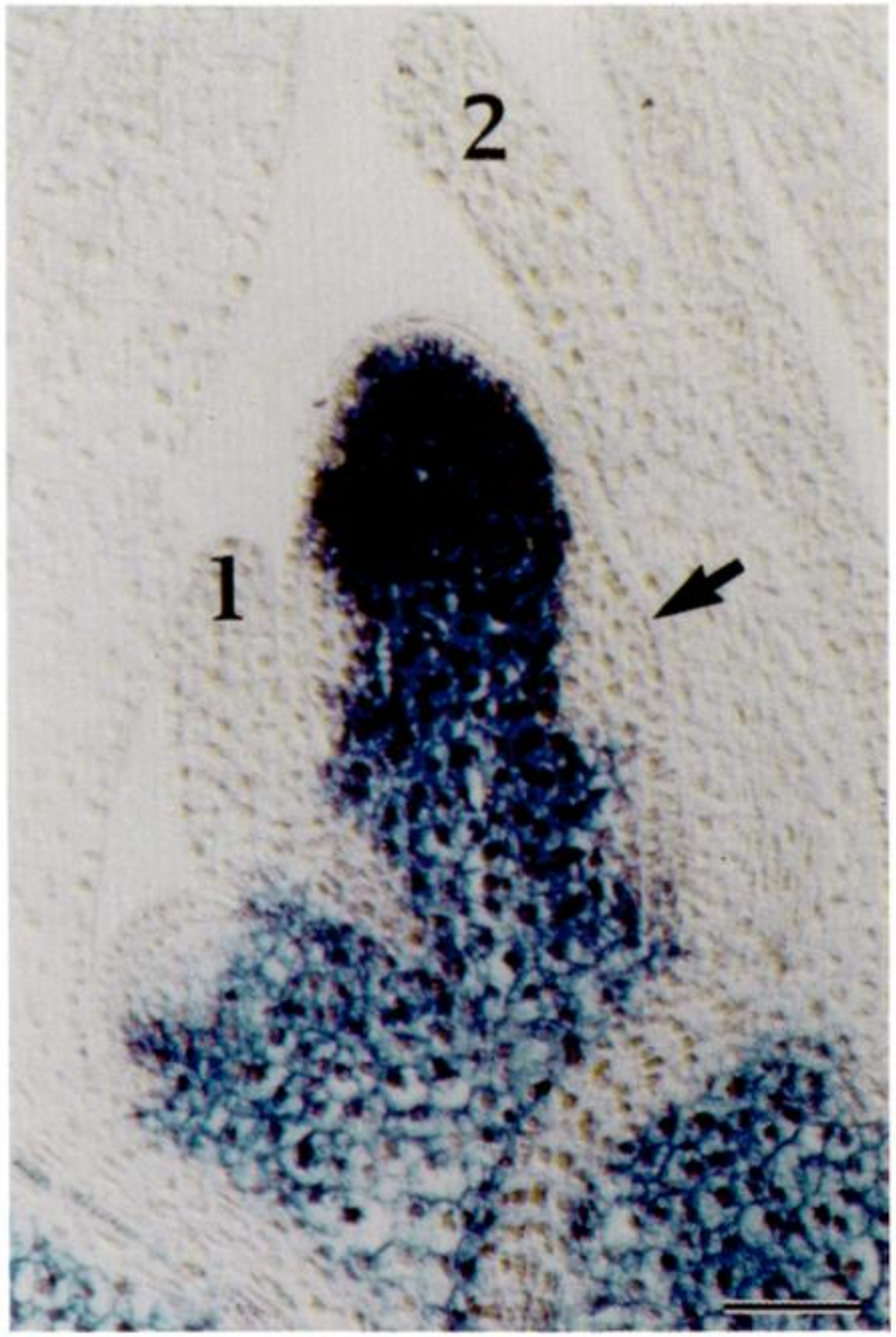
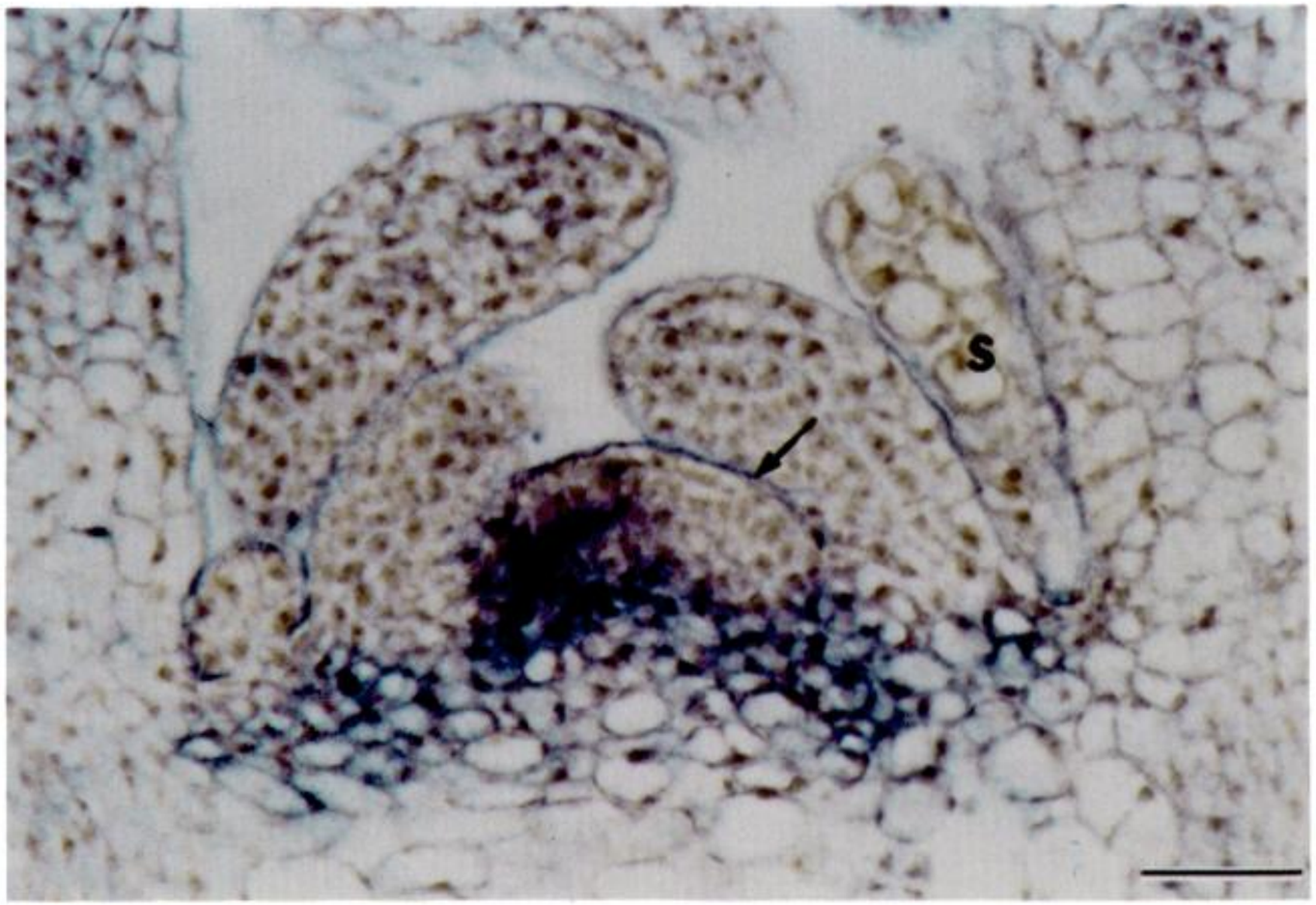
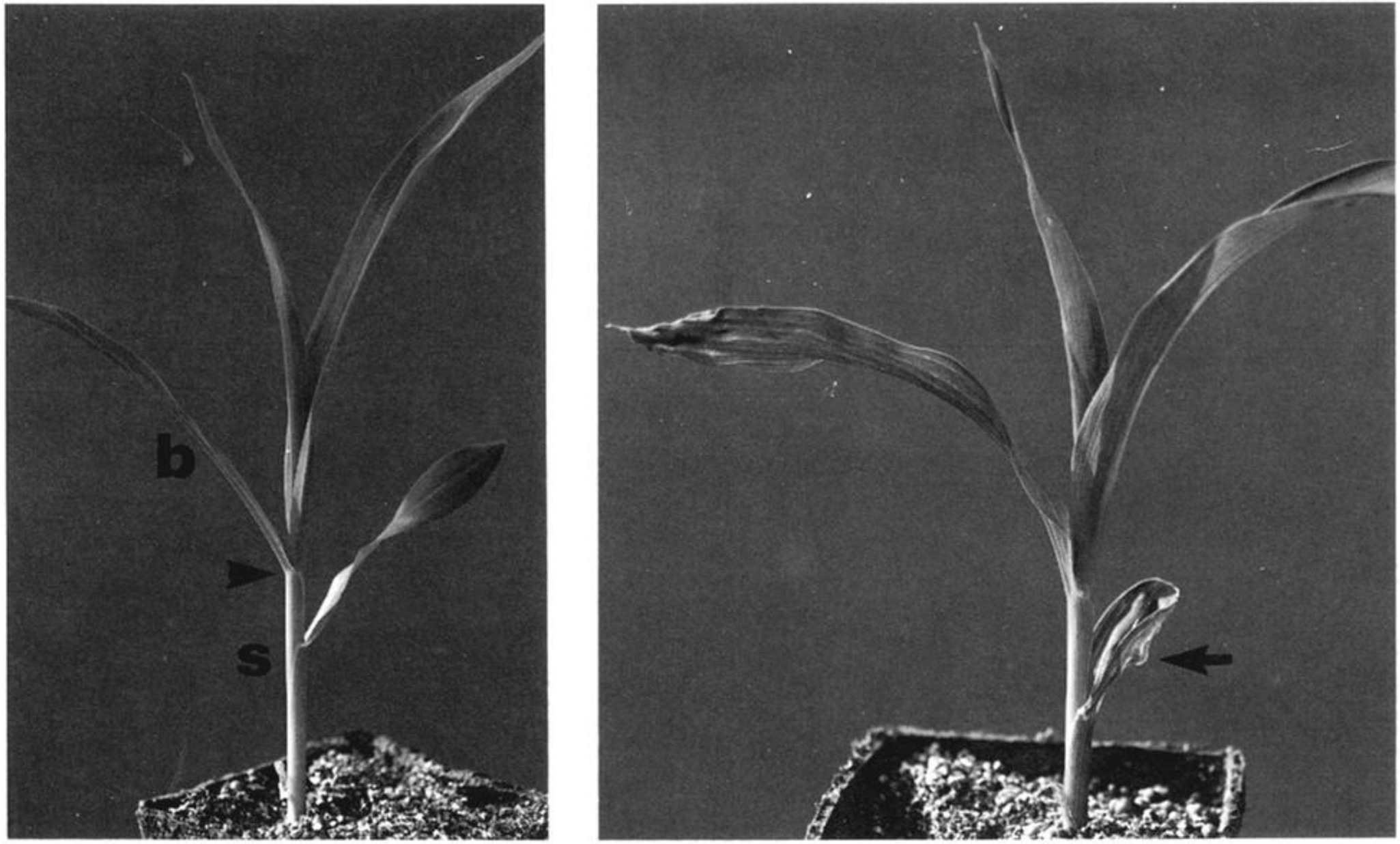
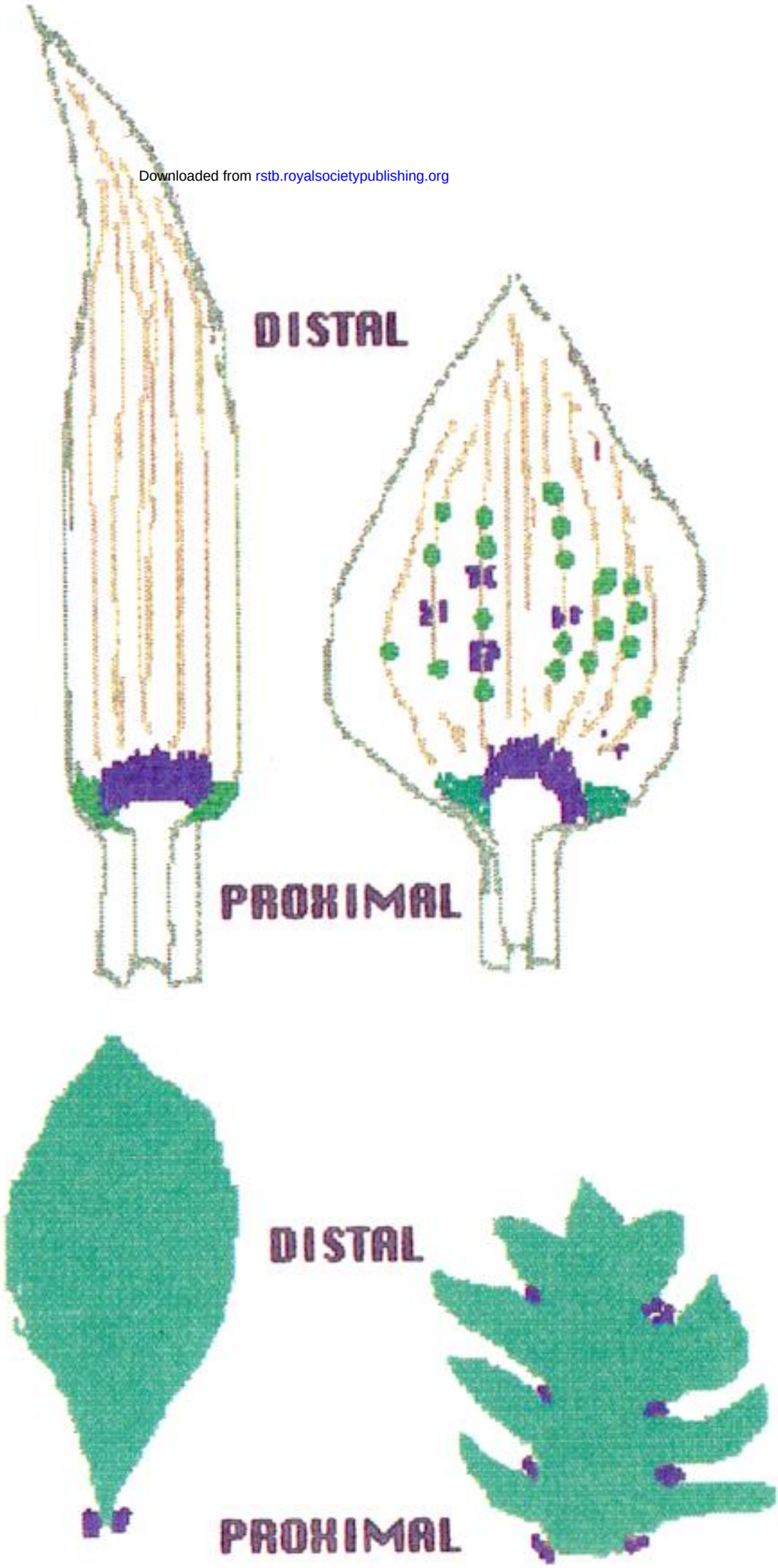




gure 2. *In situ* hybridizations of *kn1*-like genes in the vegetative meristems. *kn1* (left panel) and *knat1* (right panel) e expressed in the vegetative meristems of maize and *Arabidopsis*, respectively (Jackson *et al.* 1994; Lincoln *et al.* 94). Expression disappears before primordia formation.



gure 4. Normal and knotted seedlings. The position of the ligule is marked with an arrowhead. The blade is above e ligule and the sheath below it. The knots at the tip of the leaf are indicated with an arrow.



gure 5. Positional model for *kn1* and *knat1* gain of function
enotypes.

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