Running head: *knotted1* and leaf polarity

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Distal expression of knotted1 in maize leaves leads to re-establishment of proximal/distal patterning and leaf dissection

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ABSTRACT

Zea mays (maize) leaves provide a useful system to study how proximal/distal patterning is established because of the distinct tissues found in the distal blade and the proximal sheath. Several mutants disrupt this pattern including the dominant knotted1-like homeobox (knox) mutants. knox genes encode homeodomain proteins of the TALE superclass of transcription factors. Class I knox genes are expressed in the meristem and down-regulated as leaves initiate. Gain-of-function phenotypes result from misexpression in leaves. We identified a new dominant allele of maize knotted1, Kn1-DL, which contains a transposon insertion in the promoter in addition to a tandem duplication of the kn1 locus. In situ hybridization shows that kn1 is misexpressed in two different parts of the blade that correlate with the different phenotypes observed. When kn1 is misexpressed along the margins, flaps of sheath-like tissue form along the margins. Expression in the distal tip leads to premature termination of the midrib into a knot and leaf bifurcation. The gain-of-function phenotypes suggest that kn1 establishes proximal/distal patterning when expressed in distal locations and lead to the hypothesis that kn1 normally participates in the establishment of proximal/distal polarity in the incipient leaf.
INTRODUCTION

Plants produce organs throughout their life span from meristems, groups of self-renewing cells whose derivatives become the roots and shoots of a plant (Veit, 2006). The shoot apical meristem (SAM) initiates the leaves and stem, while the root apical meristem is responsible for generating the root system. The SAM initiates leaves in a defined pattern, called phyllotaxy, and at defined intervals, referred to as plastochrons. A leaf in plastochron 1 (P1) has just emerged from the meristem, while a P2 leaf is one plastochron older. From observing the position of these leaf primordia, one can predict the position of the next leaf (P0) while it is still part of the meristem. Given that meristem cells are indeterminate and leaf cells are determinate, a major question in plant biology is how cell fate changes during the transitions from meristem to P0 and then to leaf.

In the last decade, a number of genes have been identified that are expressed in meristems and not in leaves and thus provide insight into the process of leaf initiation. The class I knotted1-like homeobox (knox) genes are expressed throughout the SAM except in the P0 cells (Jackson et al., 1994). It has been hypothesized that down-regulation of knox genes at P0 is required for leaf initiation. Indeed, in species with dissected leaves where knox genes are expressed in leaves, knox down-regulation still occurs in the P0 (Hay and Tsiantis, 2006; Champagne et al., 2007). The importance of knox genes for plant development is highlighted by loss-of-function mutants. Plants carrying mutations in the Arabidopsis SHOOT MERISTEMLESS (STM) gene terminate after the cotyledons form, and further growth occurs from adventitious meristems that may also terminate (Long et al., 1996). The absence of a functional maize knotted1 (kn1) gene also produces a shootless phenotype in certain inbred backgrounds (Vollbrecht et al., 2000). In other backgrounds, vegetative development is normal but plants have reduced axillary branching, and ectopic leaves form in the axils of leaves (Kerstetter et al., 1997; Lunde and Hake, 2009).

Both recessive and dominant mutations have been found that cause misexpression of knox genes in leaves (Hake et al., 2004). The recessive mutants identify regulatory proteins that normally repress knox expression in leaves (Schneeberger et al., 1998; Timmermans et al., 1999; Tsiantis et al., 1999; Byrne et al., 2000; Ori et al., 2000; Scanlon et al., 2002; Ha et al., 2003; Alexander et al., 2005; Xu and Shen, 2008). Gain-of-function knox phenotypes are known in maize, barley and Antirrhinum. These dominant mutations are due to changes in cis-regulatory sequences of the knox genes and emphasize the complex regulation needed to keep knox genes off in the leaf (Greene, 1993; Greene et al., 1994; Müller et al., 1995; Schneeberger et al., 1995; Mathern and Hake, 1997; Golz et al., 2002; Uchida et al., 2007;
Guo et al., 2008). Analysis of the maize dominant knox mutants highlights a consistent theme of proximal tissues displaced into distal tissues. The timing of knox misexpression was hypothesized to be critical to the mutant phenotypes (Freeling, 1992; Muehlbauer et al., 1997).

Here we describe a new kn1 allele, Kn1-DL, which results from alterations in the promoter region. kn1 misexpression at margins of the developing blade leads to flaps of sheath tissue at blade margins. kn1 misexpressed at the midrib and tip of the blade leads to a bifurcated leaf, which is highly unusual for grass species. The Kn1-DL phenotype indicates that kn1 reinitiates new proximal/distal patterns, suggesting it may play a role in establishing proximal/distal polarity during normal leaf development.

RESULTS

Phenotype of Kn1-DL

Maize leaves have a proximal sheath that wraps around the stem, a distal blade that tilts away from the stem, and the ligule/auricle region at the junction of blade and sheath (Figure 1A, B, G) (Sylvester et al., 1990). The result of knox misexpression is a displacement of proximal tissues, such as sheath, auricle or ligule, into the blade region. This phenotype has been characterized for two other dominant alleles, Kn1-N and Kn1-O (Gelinas et al., 1969; Freeling and Hake, 1985). In Kn1-N, outpockets of tissue, called knots, occur along lateral veins of the blade (Figure 1F). The cells overlying the veins resemble those found in sheath tissue (Sinha and Hake, 1994). Occasionally, ligule tissue is found running parallel along the veins (Figure 1E). Kn1-O has a stronger effect on the ligule region, leading to a complete absence of ligule on the first leaf (Figure 1H) (Mathern and Hake, 1997). In comparison, the ligule of Kn1-N has small gaps at lateral veins (Figure 1I). On older Kn1-O leaves, knots are found toward the midrib, coinciding with displaced ligule on the adaxial side (Figure 1C, D).

Kn1-DL was identified as an individual plant in a family carrying a single MuDR and single Mu1 element (Lisch et al., 1995). The mutant has been introgressed into the W22 and B73 backgrounds for at least five generations. Similar to Kn1-N and Kn1-O, the phenotype is consistent and completely penetrant in both inbred backgrounds.

In the B73 background, the Kn1-DL phenotype is initially very mild and visible only at the margin near the tip of the blade starting with the sixth leaf (Figure 2A, B). The mildest defect is a patch of cells along the margin that rolls inward (toward the adaxial side) (Figure 2D), a phenotype never seen in margins of normal sibling leaves (Figure 2C), nor in Kn1-N or Kn1-O. These rolled patches display hairs that are similar to those visible on the abaxial surface of normal sheath margins (Figure 1B) and on the tips of knots in Kn1-N (Figure 1F).
The presence of these hairs in Kn1-DL leaf margins suggests the acquisition of sheath-like cell identity. The direction of curling, toward the adaxial surface, is the same as seen in normal sheath. Flaps are also visible along the margin (Figure 2A, B, E). These are initially less than 3-4 mm (leaf 6) and become larger and more abundant on later formed leaves (Figure 2A, B). Reorientation of the vasculature occurs at the position of the flaps (Figure 2E).

In addition to the margin flaps, the vein clearing that is common to other Kn1 alleles is occasionally detectable in lateral veins of the blade, particularly in the most distal part (Figure 2E, I, K). The “clear veins” have sheath cell identity (Sinha and Hake, 1994).

The Kn1-DL defect is also seen at the midrib. Small knots are occasionally found on young leaves that appear similar to knots on lateral veins in Kn1-N. However, on the last few leaves, a knot forms at the midrib that appears to lead to a bifurcated leaf. In a family of 13 mutants in the B73 background, the last leaf, leaf 20, was either a small flap of sheath tissue (3/13) (not shown) or bifurcated to the base of the leaf (10/13) (Figure 2F). The bifurcation was always at the position of the midrib, and tissue with sheath-like hairs could be seen along the margin created by the bifurcation (Figure 2I). Leaf 19 was bifurcated into the blade (Figure 2G), ending in a large knot, occasionally with a flap of ligule visible inside the knot (Figure 2J). The depth of this bifurcation ranged from 20 to 80% of the length of the blade. Leaf 18 was bifurcated 5 to 13% of the length of the blade (Figure 2H, K), also ending in a knot. The midribs of leaves 15-17 often ended in a small tendril, but were not bifurcated (Figure 2N). Thus, the bifurcation was more severe as the leaf position approached the tassel. Interestingly, the margins of leaves that were bifurcated showed fewer or no flaps, but were hairy and sheath-like.

The Kn1-DL defect is also visible on leaves of axillary branches. Two types of axillary branches are found in vegetative nodes of maize plants: the ear, in the axil of ~leaf 14, and tillers, which arise from basal nodes. Ears are enclosed in approximately ten husk leaves. In the B73 background, normal husks are triangular in shape and composed primarily of sheath tissue. In Kn1-DL, the distal margin of all the husk leaves is completely disrupted, and the husks do not come to a point (Figure 2L, M). In contrast, the subtending leaf in which the ear arises is only mildly affected with a few margin flaps. Tillers are rare in most inbred backgrounds, thus could not be evaluated in the introgressed Kn1-DL plants, but when they did form in mixed backgrounds, each leaf was affected (not shown).

To help determine the type of tissue present in the Kn1-DL flaps, we made hand-sections through normal and Kn1-DL leaves. In normal blades, veins are centrally located and most cells have high chloroplast content (Figure 3A). Macrohairs are adaxially located
(Sylvester et al., 1990; Candela et al., 2008). In auricle tissue, veins are also centrally located, but only a few cells contain chloroplasts, mainly on the abaxial side adjacent to the epidermis (Figure 3B). Occasional long hairs can be seen near the adaxial margin (Figure 3C). In the sheath, the vasculature is abaxial and chloroplasts are found associated with the veins. Large parenchyma cells separate the veins from the adaxial epidermis (Figure 3D). The margins of sheath have extremely long hairs (Figure 1B, 3H, 3I).

In Kn1-DL flaps, the tissue resembles sheath or auricle in many ways. The number of cells showing accumulation of chloroplasts is drastically reduced compared to normal blade. Veins are mostly abaxial (Figure 3F), though occasionally they are central (Figure 3G). Hairs are sometimes found on both adaxial and abaxial surfaces (Figure 3E), but often they are only present on the abaxial side (Figure 3F, G), as in sheath. We also sectioned through a midrib knot that was coincident with blade termination (Figure 3J, K). Compared to the midrib in a normal portion of the same leaf (Figure 3L), the region of the knot has very long hairs, similar to the sheath margin (Figure 3H, I). These hairs flank a region with hairs similar to the sheath. The vasculature and photosynthetic cells are highly abnormal.

In summary, Kn1-DL has a major effect on the distal portion of leaves. In early leaves, blade tissue is replaced by sheath/auricle-like tissue at the margins. This transformation results in the appearance of sheath hairs, flaps and curled margins. On leaves initiated late, the midvein becomes progressively more affected and terminates in a tendril or a large knot, and the leaf becomes bifurcated.

**The 5’ region is altered in Kn1-DL**

Most dominant Kn1 alleles result from transposon insertions into the third or fourth intron (Hake et al., 1989; Veit et al., 1990; Greene et al., 1994), but the Kn1-O allele results from a tandem duplication of the gene (Veit et al., 1990). We used DNA gel blots and PCR to determine the cause of the Kn1-DL mutation. Digestion with SacI and hybridization with a probe specific to the third intron showed no difference between Kn1-DL mutants and normal siblings (Supplemental Figure 1). This combination of enzyme and probe would have detected the presence of an insertion in the third intron, where 12 of the previously identified dominant alleles have transposons insertions (Hake et al., 1989; Greene et al., 1994; Ramirez, 2007). In contrast, digesting the DNA with BclI, which cleaves upstream, downstream and within the coding region (Figure 4A), produced a unique hybridization pattern in Southern blots. Using a probe spanning the two BclI sites within the coding region, we found that the 0.5 Kb fragment that spans the fourth exon and the 3.1 Kb fragment that extends into the 3’ end are the same in
mutants and normal siblings, but the 9.1 Kb fragment that extends into the 5’ end is replaced by two fragments, a 6.2 and >12 Kb fragment (Figure 4B). The 9.1 Kb fragment is the same size as the band detected with DNA from normal siblings, the progenitor (from which the Kn1-DL mutant originated), and all the inbred backgrounds tested (data not shown). Finding two bands instead of one suggests Kn1-DL contains a partial or complete duplication of the kn1 locus that disrupts the 5’ BclI site. We compared the BclI digested DNA from Kn1-DL plants with that from Kn1-O using a probe specific to the third intron. The DNA from Kn1-O generated two bands, one in common with the normal siblings and another band slightly smaller (Figure 4C). Thus, the duplications found in Kn1-O and Kn1-DL are distinct. To confirm that the duplication is linked to kn1, we carried out DNA gel blot hybridization with 75 Kn1-DL individuals and found that they all produced the same pattern (data not shown), indicating that the duplication is closely linked to the phenotype. Moreover, because fragments in the 3’ end of the kn1 locus are identical in normal and Kn1-DL, the duplication is likely to be 5’ of the gene.

Because Kn1-DL arose in a line carrying active Mutator (Mu) elements, we used PCR to determine if a Mu element could be identified in the Kn1-DL allele. We were able to amplify a fragment using a Mu primer, which recognizes the terminal inverted repeats common to all Mu elements, and a primer designed from the 5’ region (Figure 4E). This fragment was only detected using DNA from knotted individuals but not from normal siblings or progenitor. We localized the Mu element 297 bp upstream of the start codon. Primers flanking this Mu insertion were able to amplify a fragment in normal siblings and heterozygotes, but not presumed homozygotes (Figure 4E) nor homozygotes identified by Southern blot. The Mu primer in combination with primers up to ~2.5 Kb upstream of the start codon amplified the expected DNA fragments as verified by sequencing. Thus, it is likely that duplicated elements of the kn1 locus are located upstream of the kn1 promoter.

The phenotype of dominant Knl alleles resulting from Mu insertions depend on the presence of an active transposase, MuDR (Greene et al., 1994). We crossed Kn1-DL and other Mu-containing Knl alleles to MuKiller, a line of maize that suppresses Mu activity (Slotkin et al., 2005). MuKiller was able to effectively suppress the knotted phenotype in all other Mu-containing alleles except Kn1-DL (Table 1). Either the Mu insertion at Kn1-DL is unique, or the cause of kn1 misexpression is not only the Mu element, but also the rearrangement resulting from the duplication.
mRNA accumulation in leaves

To determine the size of the mRNA in Kn1-DL, we carried out RNA gel blot analysis. We detected in Kn1-DL leaf primordia a band of the same size as the one detected in meristem-enriched tissues of normal or mutant plants (Figure 4D). Similarly, RT-PCR performed with a primer pair able to amplify a nearly full length cDNA showed a band of the expected size (Figure 4F). The same primer pair was used to determine when ectopic knl transcripts could be detected in leaf tissue of Kn1-DL. When using leaves dissected from 10-day-old seedlings, knl cDNA was detected in Kn1-N but not Kn1-DL nor B73 after 33 cycles of PCR, but a faint band was detected in Kn1-DL after 40 cycles of PCR. In our growth conditions, those seedlings had two visible leaves with the third just emerging, thus the tissue harvested included mainly developing leaf primordia three and four. knl cDNA was easier to detect with 18-day-old Kn1-DL seedlings, which included leaf 5 and younger, i.e., subsequently initiated leaves, and 26-day-old seedlings, which included leaf 7 and younger. Accumulation of knl cDNA in 26-day-old Kn1-DL leaves was similar to the accumulation detected in Kn1-O/knl+ plants of the same age. Thus, the timing of expression coincides with the appearance of the phenotype. Indeed, all leaves from the Kn1-N mutant show the knotted phenotype, whereas only later leaves from Kn1-DL mutants show the phenotype.

To investigate the spatial pattern of knl mRNA accumulation, we carried out in situ hybridization. As described previously, the Kn1-N defect is restricted to lateral veins of the leaf blade (Figure 1F). Our analysis of Kn1-N shoot apices confirmed previous in situ results (Smith et al., 1992; Jackson et al., 1994), detecting the mRNA in lateral veins of blades but not sheaths (Figure 5B). No signal could be detected in leaves of normal siblings (Figure 5A).

In order to examine the expression of knl in Kn1-DL leaf primordia, we sectioned shoot apices of plants that had just transitioned to flowering, thus allowing us to visualize knl accumulation in the last leaves initiated by the SAM, which show the most severe phenotype. The most basal section in the series in Figure 5 shows expression of knl in the SAM but not in leaves, with the exception of a small dot of expression in a margin of the leaf 17 (Figure 5C). A few sections above (Figure 5D), strong knl accumulation is detected in a tassel branch as well as in many leaves. knl expression in leaves 15-19 is visible mainly at the margins. Expression in leaf 20 is scattered throughout the leaf, including around the midrib. These leaves did not show any knl expression at their most basal part (Figure 5C). Figure 5E shows knl expression in two tassel branches and at the margin of leaves 17 and 18, and throughout leaf 19 including the midrib. Leaf 20 is not visible in this section, and thus is a very young leaf. Figure 5F and 5I show expression throughout leaf 18 and at leaf margins. Leaf 19 is no
longer visible. In Figure 5G, expression is only detected at margins for leaves 15-17 while leaf 18 primordium is no longer visible. In the last panel of the series, 1-2 mm above the tip of the tassel primordia, \( knl \) expression is mainly at margins. Thus, \( knl \) is primarily misexpressed in the distal part of leaf margins except in the upper three leaves, where expression occurs throughout the tip of the leaf primordia.

**DISCUSSION**

We identified a new dominant allele of \( knl \), \( Kn1-DL \). Our data indicate that \( Kn1-DL \) arose from a \( Mu \) insertion in the promoter as well as a duplication of part of the locus. The coding region is not affected. \( Mu \) insertions have been shown to result in rearrangements at \( Knl-O \) (Mathern and Hake, 1997) as well as other loci (Stinard et al., 1993), and thus it is probable that the duplication event found in \( Kn1-DL \) arose after the \( Mu \) insertion. The phenotype in \( Kn1-DL \) is progressively more severe with plant age. *In situ* hybridization shows that \( knl \) is misexpressed in two different parts of the blade correlating with the different phenotypes observed. When \( knl \) is misexpressed along the margin, it favors the formation of flaps of sheath-like tissue. Expression in the distal tip of the last few leaves leads to premature termination of the midrib into a knot and leaf bifurcation. These phenotypes and expression patterns allow us to formulate a hypothesis for the function of \( knl \) in normal leaf initiation.

Early events in leaf initiation include polarization of PIN proteins with an increase in auxin response, as reflected by the expression of the auxin reporter DR5 (Reinhardt et al., 2003; Heisler et al., 2005; Bayer et al., 2009). Genes that regulate abaxial/adaxial polarity, such as class III HD-ZIP transcription factors, are also expressed in the P0 (McConnell et al., 2001; Juarez et al., 2004), and a current model proposes that adaxial/abaxial polarity is established at this early stage (Chitwood et al., 2007). An additional marker of the P0 is the down-regulation of \( knox \) mRNA (Jackson et al., 1994). We propose that proximal/distal polarity may also be initiated in the P0 and that KN1 participates in this process (Figure 6A, B). \( knl \) mRNA is present in meristem cells, excluding the P0. However, because KN1 protein moves, it is found in cells at the boundary between meristem and P0 (Jackson, 2002), coinciding with the proximal end of the leaf. Thus, the absence of KN1 in most P0 cells leads to cell division and differentiation in the leaf primordium, but its presence at the boundary of P0 and meristem retards these activities, possibly through negative regulation of GA (Hay et al., 2002; Jasinski et al., 2005; Bolduc and Hake, 2009).
If knl establishes proximal/distal patterning, what happens when it is missing? Depending on inbred, knl loss-of-function mutants may terminate with a coleoptile or one or two leaves (Vollbrecht et al., 2000). In the embryo, a flattened zone of differentiated cells is found where the meristem would normally reside. The failure to make leaves has been attributed to a failure to maintain the meristem. It is also possible that leaves do not form because knl is required to establish the proximal boundary of the leaf. A similar failure to initiate leaves can occur when there is loss of adaxial fate identity following overexpression of genes that specify abaxial fate (Eshed et al., 2001). Indeed, ectopic leaf flaps develop when adaxial cells are found in a field of abaxial cells (Waites and Hudson, 1995; Timmermans et al., 1998; Candela et al., 2008). Considering the knl loss-of-function and gain-of-function mutant phenotypes, a similar juxtaposition of proximal and distal cells may be required to elaborate a leaf. The expression of knl at the margins of the leaf in Kn1-DL creates a juxtaposition of proximal cells (knl-expressing) with distal cells (the blade). This juxtaposition reiterates the proximal/distal patterning that normally occurs in the Po, leading to a reprogramming of proximal/distal polarity and the formation of leaf flaps (Figure 6E). knl misexpression in the lateral veins of Kn1-N blades allows these cells to adopt more proximal cell identities, but due to the constraints by blade cells on either side, the sheath-like cells grow out of the plane of a leaf, making a knot (Figure 6D). We speculate that the correct balance of abaxial with adaxial as well as proximal with distal determinants is required for normal leaf initiation, while inappropriate juxtaposition of proximal with distal or adaxial with abaxial cell types leads to ectopic leaf flaps.

In contrast to the knl expression patterns in Kn1-N and Kn1-DL, misexpression seen in other dominant knox mutants appears to be continuous with the meristem. In Gnarley, expression extends from the meristem up into the proximal end of early plastochron leaves (Foster et al., 1999). In Roughsheath1 (Rsl), misexpression is seen in P1 and P2 leaves (Schneeberger et al., 1995). In the dominant Lg4 mutant, misexpression of liguleless4 (lg4) was found by RT-PCR in the sheath but not in the blade (Muehlbauer et al., 1999). These three mutants have no ectopic leaf flaps or knots in the blade; rather, the boundary between sheath and blade is displaced toward the distal part of the leaf (Figure 6C). Thus, we propose that new proximal/distal patterning takes place when misexpression of knox genes occurs in distal tissues whereas misexpression in proximal tissues, contiguous with the meristem, displaces boundaries, but does not create new ones.

Aspects of the Kn1-DL phenotype are shared by other maize mutants. Leaves on leafbladeless1 (lbl1) mutants are often transformed into radially symmetrical tendrils.
(Timmermans et al., 1998). These narrow leaves are due to smaller groups of leaf initial cells, i.e. fewer cells in which knl expression is down-regulated. Occasionally, on more normal leaves, a knot is present in association with a bifurcation of the leaf somewhat similar to Knl-DL (Timmermans et al., 1998). lbl1 encodes a protein in the trans-acting siRNA pathway that promotes adaxial fate (Nogueira et al., 2007). It is possible that misexpression of knl at the midvein interferes with abaxial/adaxial polarity establishment, leading to the tendrils seen at the tips of Knl-DL leaves. Likewise, the loss of adaxial fate in lbl1 mutants may interfere with proximal/distal establishment, thus causing the bifurcation. Interplay between adaxial/abaxial and proximal/distal axes has been previously documented (Ha et al., 2004; Candela et al., 2008).

Two other dominant maize mutants that make flaps are Lax midrib1-O (Lxm1-O) and Hairy sheath frayed1-O (Hsf1-O). Similar to some knox mutants, the boundary between sheath and blade of Lxm1-O mutants is displaced toward the distal part of the leaf. The flaps of Lxm1-O mutants, however, are distinct from those of Knl-DL, as they form on the abaxial side of the leaf adjacent to the midrib (Schichnes et al., 1997). Flaps that form in the blade have blade identity/characteristics, while flaps that form in the sheath have sheath identity/characteristics. Thus, these flaps are not likely to be due to new proximal/distal patterning. Indeed, knl misexpression was not detected in Lxm1-O (Schichnes et al., 1997). The flaps of Hsf1-O are similar to those of Knl-DL in that they occur on the margins of blades and have sheath characteristics. Unlike Knl-DL flaps, which grow out of a symmetrical leaf, the Hsf1-O flaps are found in combination with a narrowing of the blade, as if cells were diverted into the flap (Bertrand-Garcia and Freeling, 1991). The Hsf1-O mutation is very pleiotropic and affects all stages of development and most tissues. Cloning of both lxm1 and hsf1 will provide an understanding of how these pathways intersect.

Misexpression of knox genes leads to leaf dissection in Arabidopsis (Lincoln et al., 1994; Chuck et al., 1996) and increases leaf dissection in tomato (Hareven et al., 1996; Chen et al., 1997). Consistent with those findings, lowering and raising knox expression in Cardamine hirsuta, a member of the Brassica family with dissected leaves, affects leaf shape. RNAi lines with reduced amounts of the C. hirsuta STM ortholog show a reduction in number of leaf lobes. In addition, C. hirsuta plants that express knl through an inducible KN1-GR construct show increased leaflets as well as a shorter proximal distal axis (Hay and Tsiantis, 2006). We propose that the misexpression of knl at the distal tip of Knl-DL is responsible for dissection on the upper leaves (Figure 6F). Given that ectopic knl mRNA increases with time in Knl-DL, the severity at the midrib is likely due to increasing amounts of KN1 at the tip of
the leaf. Although these leaves are unusual, they reveal that grasses are not completely immune to architectural restructuring by knox misexpression.

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MATERIALS AND METHODS

Genomic DNA analysis

Isolation of genomic DNA and DNA gel blots was carried out using previously published protocols (Lowe et al., 1992). Probes for Southern blots were generated using PCR with the following primers: Kn1-F58 (TTATTTCCGTCGTTCTGAG) + Kn1-R1372 (GGAAGGAGAGGGAAAGGAGA), E25 (TCTTGGATCGGTTGTCGCCT) + Kn1-R5682 (TTGACAACATTTGGGACAA), and Kn1-7128 (GGGCAACTTCACATGTCCTT) + Kn1-R8614 (ATGTAGAAGGCATTGGTGGTG). Mu-specific primer (AGAGAAGCCAACGCAWCGCTCYATTTGCT) was used in combination with NB-357F (AAAAAGAAGCCCCACAAAAC) and NB-341R (CGCCTGGAGATGAGATGAAG). The identity of amplified DNA was verified by sub-cloning into pGEM T-Easy vector (Promega) and sequencing. The identity of amplified DNA was verified by sub-cloning into pGEM T-Easy vector (Promega) and sequencing.

RNA Analysis

For Northern blot analysis, total RNA was isolated from two to three week-old seedlings using Trizol reagent according to the manufacturer’s instructions (Invitrogen). Ten μg of total RNA were glyoxylated, separated by electrophoresis in a 1.2% agarose gel and transferred to nylon membrane as previously described (Smith et al., 1992). Hybridizations were performed with the full kn1 cDNA (Vollbrecht et al., 1991). Following autoradiography, filters were stripped and re-probed with an ubiquitin probe to assess RNA quality and quantity.

For RT-PCR analysis, total RNA was further purified with Qiagen RNeasy Mini Kit following the manufacturer’s recommendations for RNA cleanup. Reverse transcriptions were
performed at 46°C for three hours using 2 µg of total RNA, SuperscriptIII (Invitrogen) and oligo(dT) primers. PCR were then performed with 1 µl of cDNA using kn1-specific primers E47 (GAGATCACCCAACACTTTGG) and Kn1-3’R (ACATGAGCGGTACCATTAGATTAGG) and actin-specific primers ActinF (AAGTACCCGATTGAGCATGG) and ActinR (GATGGAGTTGTACGTGGCCT).

**Histology and in situ hybridization**

For histology analysis, hand-sections were mounted in 50% glycerol and viewed immediately using dark-field microscopy. For in situ hybridization, shoot apices of three to four week old seedlings were fixed overnight with FAA (50% ethanol, 5% glacial acetic acid, 3.7% formaldehyde, 0.5% Triton X-100, and 1% DMSO) and embedded in paraffin after staining with 0.5% (w/v) Eosin Y in ethanol as previously described (Jackson, 1992). Tissue sections were pre-treated and hybridized as previously described (Jackson et al., 1994).

**SUPPLEMENTAL MATERIAL**

**Supplemental Figure 1:** Southern blot analysis of Kn1-DL DNA with SacI and BclI. A) Map of kn1 locus identifying the exons (black boxes) and restriction enzymes sites. The bar under the third intron shows the position of the probe used in (B). The triangle shows the location of the Mu element in Kn1-DL. B) Southern blot of DNA from Kn1-DL and normal siblings cut with SacI or BclI.

n, normal sibling; Kn, Kn1-DL/+.

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FIGURE LEGENDS

Figure 1. Phenotype of Kn1-N and Kn1-O. A) Maize leaves have a sheath that wraps tightly around the stem and a blade that is flat. B) The boundary of blade and sheath consists of a ligule (an epidermal fringe on the adaxial surface), and triangular shaped auricles. C) Adaxial surface of Kn1-O leaf 12. Ectopic and displaced ligule is found in the blade region. D) Knots on the abaxial surface of leaf in (C). E) Paired ligule fringes on adaxial surface of Kn1-N leaf blade. F) Abaxial surface of Kn1-N blade. The knots are localized to the lateral veins. Insert highlights hairs over the knots that resemble sheath or auricle hairs. G-H) Close-up of the ligule region in leaf 1 from a normal sibling (G), Kn1-O (H) and Kn1-N (I). au, auricle; sh, sheath; bl, blade; lg, ligule.

Figure 2. Phenotype of Kn1-DL in B73. A-B) Flaps in Kn1-DL leaves. A) From left to right: normal sibling leaf 6, Kn1-DL leaf 6, normal sibling leaf 11, Kn1-DL leaf 11. B) Close-up of Kn1-DL leaf 6. Arrows point to same positions as in (A). C) Margin of normal sibling leaf 6. D) Margin of Kn1-DL leaf 6 with close-up in inset, showing hairs typical of sheath tissue. E) Small flap on Kn1-DL leaf showing hairs and reorientation of veins. F-H) Consecutive leaves from a Kn1-DL plant. F) Leaf 20 (last leaf). G) Leaf 19. H) Leaf 18. I) Close-up of margin from leaf in (F) showing hairs and reorientation of veins. J) Close-up of adaxial surface of midrib knot from (G). Notice the presence of ectopic ligule (arrow). K) Close-up of abaxial surface of midrib knot in (H). L) Ears from a normal sibling (left) and a Kn1-DL plant (right). The distal part of each husk leaf is affected. M) Distal portion of normal (left) and Kn1-DL (right) husk leaves taken from ears in (L). N) Cleared leaf 15 from Kn1-DL showing midrib that ends in tendril.

Figure 3. Histology of Kn1-DL. A-I and K-L are hand sections visualized under dark-field microscopy. All sections are adaxial side up except when noted. All pictures were taken at the same magnification except I and J. A) B73 blade. B) B73 auricle. C) B73 auricle margin. D) B73 sheath. E-G) Leaf flaps from Kn1-DL. H-I) B73 sheath margin. Abaxial side is to the left in (H) and to the top in (I). J) Knot at the midrib of a Kn1-DL plant. Arrow points to the region of the section in (K). K) Hand-section through the knot in (J). Note the long hairs that are similar to the hairs of the normal sheath margin. L) Same midrib as in (J) and (K), but in normal region of the leaf.
**Figure 4.** Molecular analysis of Kn1-DL. A) Map of knl locus identifying the exons (black boxes) and restriction enzymes sites. The gray bar under the fourth and fifth exons shows the position of the probe used in (B), while the black bar under the third intron shows the position of the probe used in (C). The triangle shows the location of the Mu element detected in Kn1-DL. The arrows flanking the Mu element show the position of primers used in (E), while arrowheads show the position of primers used in (F). B) Southern blot of DNA from Kn1-DL and normal siblings cut with BclI. C) Southern blot comparing Kn1-DL with Kn1-O. The DNA was digested with BclI. D) Northern blot comparing Kn1-DL and normal siblings leaf primordia RNA after hybridization with the full length knl cDNA. The size of the detected mRNA is the same regardless of the genotype and tissue. knl is detected in Kn1-DL leaves (arrow) but not normal siblings. A higher molecular weight band is seen in both leaf samples, but is believed to be non-specific. E) Detection of the Mu element in the knl promoter in a family segregating Kn1-DL. Asterisks identify Kn1-DL individuals presumed to be homozygotes based on the failure to amplify across the Mu element with F and R primers. F) RT-PCR analysis of knl mRNA in leaf primordia. The two upper panels were performed with knl-specific primers and the lower panel with actin specific primers. N, normal sibling; Kn, Kn1-DL.

**Figure 5.** In situ hybridization of knl in leaf primordia. A) No expression is detected in B73 leaves. B) Expression is in punctuate pattern around veins in Kn1-N blades. C-H) Serial sections through a Kn1-DL shoot apex that has just started the transition to flowering (four weeks old) captures the last few leaves that were initiated by the SAM. Sections are shown from the base of the tassel (C) up to the leaf primordia just above the top of the tassel (H). Arrow in (C) points to expression in the margin of leaf 17. I) Close-up from (F) showing the midrib region of leaf 18 and the margins of leaf 17.

**Figure 6.** Cartoon representation of knl expression patterns and phenotypes. A) In the shoot apical meristem, knl mRNA (light blue) is detected in the meristem, but not the P0 (yellow). knl marks the most proximal (P) boundary of the P0 leaf. Similar to the adaxial (Ad)/abaxial (Ab) axis, the proximal/distal (D) axis is presumably already established in the P0. B-F) The expression pattern of knox genes in young leaf primordia is diagrammed on the left and the leaf phenotype at maturity is shown on the right. B) In a normal leaf primordium, knox expression is only at the base, at the site of leaf insertion, the most proximal end of the leaf. The mature leaf has clearly defined proximal sheath (dark blue), distal blade (orange) and
ligule (green) at the boundary between sheath and blade. C) In Gn1-R, knox expression is expanded up from the base of the leaf. The consequence is a displaced blade/sheath boundary in the mature leaf. D) In Kn1-N, kn1 expression occurs along lateral veins in the blade. Knots, composed of sheath-like cells, develop along the veins. The veins are wider and contain sheath-like cells. Ectopic ligule is sometimes visible along the veins. E) In most leaves of Kn1-DL, kn1 is expressed at margins in the distal part of the primordium. At maturity, flaps of sheath-like tissue are visible at margins of the most distal part of the blade. F) In the last few leaves of Kn1-DL, kn1 expression expands throughout the distal tip, including the midvein, in addition to the margins. A knot forms at the midrib and growth stops at this midpoint. Continued growth on either side leads to a bifurcated leaf. Ad, adaxial; Ab, abaxial; P, proximal; D, distal.
Table 1. Penetrance of Kn1

| Families                  | Genotype                               | Phenotype                      |
|---------------------------|----------------------------------------|--------------------------------|
| SH6157-8xJR90-29          | Muk/Muk x Kn1-mum1/+                  | 18 normal                      |
| JR119-3xJR90-29           | MuDR x Kn1-mum1/+; MuDR               | 20 normal: 17 knotted          |
| SH6157-3xJR97-4           | Muk/Muk x Kn1-mum2/+                  | 20 normal                      |
| JR119-13xJR97-4           | MuDR x Kn1-mum2/+; MuDR               | 17 normal: 21 knotted          |
| JR99-10xSH6157-3          | Kn1-mum8/+; MuDR x Muk/Muk            | 36 normal                      |
| JR99-10xJR119-12          | Kn1-mum8/+; MuDR x MuDR               | 16 normal: 12 knotted          |
| JR81-8xSH6157-3           | Kn1-mum9/+; MuDR x Muk/Muk            | 25 normal                      |
| JR85-4xJR81-8             | MuDR x Kn1-mum9/+; MuDR               | 11 normal: 16 knotted          |
| JR107-12xJR118-2          | Kn1-mum10/+; MuDR x Muk/Muk           | 28 normal                      |
| JR119-1xJR107-12          | MuDR x Kn1-mum10/+; MuDR              | 13 normal: 10 knotted          |
| JR118-2xJR109-20          | Muk/Muk x Kn1-mum5/+; MuDR            | 62 normal                      |
| JR109-20xJR119-7          | Kn1-mum5/+; MuDR x MuDR               | 24 normal: 34 knotted          |
| JR118-1xJR82-11           | Muk/Muk x Kn1-mum11/kn1-del           | 24 normal                      |
| JR119-12xJR82-11          | MuDR x Kn1-mum11/kn1-del              | 9 normal: 12 knotted           |
| JR85-6xJR76-9             | MuDR x Kn1-DL/Kn1-DL                  | 14 knotted                     |
| SH6157-1xJR76-9           | Muk/Muk x Kn1-DL/Kn1-DL               | 20 knotted                     |

Plants with the knotted phenotype were crossed to a line carrying MuKiller activity (Slotkin et al., 2005) and to a line carrying the active Mu tranposase, MuDR (Lisch et al., 1995). Progeny were grown to maturity and screened for knots. kn1-del is a line that carries a deletion of the kn1 locus and does not transmit through the male. Kn1-mum1, Kn1-mum2, Kn1-mum8 contain a Mu element in the third intron (Greene et al., 1994), Kn1-mum9 and Kn1-mum11 contain a Mu element in the 5’ region (Ramirez, 2007).
A) Shoot apical meristem.

B) Normal

C) *Gn1-R*

D) *Kn1-N*

E) *Kn1-DL* (leaf 6-17)

F) *Kn1-DL* (leaf 18-20)

- **knox accumulation**
- **leaf primordium**
- **blade**
- **ligule**
- **sheath**

Distal Blade

Ligule/Auricle

Proximal Sheath