The **Liguleless narrow** mutation affects proximal-distal signaling and leaf growth

Jihyun Moon¹, Héctor Candela² and Sarah Hake¹,³,*

**SUMMARY**

How cells acquire competence to differentiate according to position is an essential question in developmental biology. Maize leaves provide a unique opportunity to study positional information. In the developing leaf primordium, a line is drawn across a field of seemingly identical cells. Above the line, the cells become blade, below the line the cells become sheath and at the line, the cells differentiate into the specialized tissues of ligule and auricle. We identified a new mutation, **Liguleless narrow** (*Lgn*), that affects this patterning and shows striking defects in lateral growth as well, thus linking proximal-distal patterning to medial-lateral growth. In characterizing the defect we discovered that both the auxin transport protein ZmPIN1a and the squamosa promoter-binding protein LIGULELESS1 are expressed precisely at this positionally cued line and are disrupted by *Lgn*. Positional cloning and a transposon-derived allele demonstrate that LGN is a kinase. These results suggest that LGN participates in setting up positional information through a signaling cascade. Interestingly, LGN has a paralog that is upregulated in the mutant, suggesting an important feedback mechanism involved in setting the positional boundary.

**KEY WORDS:** PIN1, Leaf development, Maize, Pattern establishment, Preligule

**INTRODUCTION**

Leaves continuously develop at the flanks of an active shoot apical meristem, where a population of pluripotent stem cells resides. Starting as a bulge on the flank of the meristem, the newly initiated leaf becomes asymmetric in several axes: adaxial-abaxial, medial-lateral and proximal-distal. Polarization along these axes leads to the asymmetric distribution of cell types in the mature organ (Eshed et al., 2001; Moon and Hake, 2011). Maize serves as an excellent model system for the study of leaf development due to the well-organized pattern of division during leaf development and the distinct tissue types in the mature leaf (Fig. 1A). Along the proximal-distal axis, the sheath is proximal, the blade is distal and a ligular region, which consists of the ligule and auricle, is at the blade-sheath boundary. The midrib and margins of each leaf constitute the medial-lateral axis. Adaxial and abaxial epidermal surfaces have different distributions of cell types. Mutations affecting one or more of the developmental axes disrupt the patterning of the leaf, leading to a change in cell identity at specific regions.

The ligule is an adaxial structure found at the blade-sheath boundary of most grass leaves. The main function of a ligule is to repel water from entering the space between the leaf sheath and stem, where auxiliary buds form. Two recessive mutants, **liguleless1** (*lg1*) and *lg2*, provide important information in deciphering the genetic basis of leaf patterning in maize (Becraft and Freeing, 1991; Becraft et al., 1990; Harper and Freeing, 1996; Moreno et al., 1997; Sylvester et al., 1990; Walsh et al., 1998). The *lg1-R* mutation removes the ligule and auricle from most of the leaves, but the blade-sheath boundary remains distinct (Sylvester et al., 1990). *lg2-R* mutants lack ligules and auricles only on the first initiated leaves. In later-formed leaves, ligule and auricle appear at both leaf margins but the position is displaced relative to one another. *lg1* encodes a squamosa promoter-binding protein that is expressed at the ligular region (Moreno et al., 1997). *lg2* encodes a basic leucine zipper protein (bZIP) and its mRNA is expressed more broadly (Walsh et al., 1998). Mosaic analysis has shown that the *lg1-R* phenotype is cell autonomous whereas *lg2-R* is non-cell-autonomous (Becraft and Freeing, 1991; Harper and Freeing, 1996; Moreno et al., 1997). These results together lead to a proposal that LG2 acts by regulating the ‘make ligule/auricle’ signal, whereas LG1 might be involved in reception and/or downstream signal transduction of the signal (Walsh et al., 1998).

Here, we describe the characterization and cloning of the semi-dominant **Liguleless narrow**—Reference (**Lgn-R**) mutant. In **Lgn-R** heterozygotes, mRNA levels of *lg1* and *lg2* are reduced and boundary formation fails towards the margins of the leaf. Leaves are narrow and branching in the inflorescence is reduced, suggesting a link between proximal-distal patterning and lateral growth. Homozygous **Lgn-R** plants fail to produce reproductive structures and remain in a juvenile state. We mapped *Lgn* to a kinase and showed that the **Lgn-R** mutation results in decreased kinase activity and increased mRNA levels of its paralog, *sister of liguleless narrow* (**sln**). Together, these results suggest that the regulation of **Lgn** and **sln** is crucial for establishing positional cues that have major impacts on developmental processes.

**MATERIALS AND METHODS**

**Plant materials**

*Lgn-R* was found in an ethyl methanesulfonate (EMS) M1 screen in the B73 background performed by M. G. Neuffer (University of Missouri, Columbia). The chimera plant was crossed to A632, then introgressed five to six generations into inbreds including B73, W22 and W23. The *tac905.29* allele is an *Ac* insertion line generated by the Dooner laboratory (Cowperthwaite et al., 2002). Seeds (Co-op ID: T3211H) were obtained from the Maize Genetics Cooperation Stock Center (http://maizecoop.cropsci.uiuc.edu/). *lg1-R* in W23 background was used.
an undefined background. ZmPIN1a-YFP lines were described previously (Gallavotti et al., 2008).

All plants were done on the sixth leaf counting down from the top after the tassel had emerged. Ten plants were measured per genotype. The numbers were averaged and standard deviation was calculated. Blade width was measured across the midrib from the ligule to the tip and blade width was measured across the half-point of the leaf between the blade tip and ligule region. The whole-plant height was measured from the ground to the highest node beneath the tissue.

Epidermal surface replicate
Epidermal surface of the leaves were observed by the modified replica technique (Foster et al., 2004). Casts of epidermal surfaces were generated by applying dental impression medium to the leaf surface. Colorless nail polish was applied to the cast and left to dry overnight. Dried nail polish replicas were peeled off from the cast and placed on a microscope slide with water. Coverslips were mounted and the specimens were viewed under a Zeiss Axioskop differential interference contrast (DIC) microscope.

Positional cloning of lgn
F1 plants of Lgn-R in B73 crossed to selected inbreds (Mo17, A632 and W22) were backcrossed to that inbred to generate mapping populations. Genetic linkage of Lgn-R to chromosome 9 was first detected by bulked segregant analysis (Michelmore et al., 1991) using simple sequence repeat (SSR) markers on different maize chromosomes. Fine mapping was carried out by using MUSCLE software (Edgar, 2004). The phylogenetic tree was constructed based on the alignment using Phylogeny.fr (Dereeper et al., 2008).

Autophosphorylation assay
In order to express the kinase domain of the wild-type and mutant forms of Lgn, cDNA corresponding to the kinase domain was amplified and cloned into pGEX-5X-2 (GE Life Sciences). For the kinase domain amplification, JM77F, 5’-CCCGAATTCTCATCGCGGTTCGATCATGG-3’; jm89F, JM89F, 5’-CAACATCGGAGATCCT-3’ and JM89R, 5’-CTTTGGCTTGAGAAATTT-3’; snh, JM85F, 5’-ATGGAGGAACACACATGGC-3’ and JM85R, 5’-GATAGCAGGCGCCGAAACCG-3’; lgi1, lgi1-3’F, 5’-CCTAAGACAAAGCAGAGAG-3’ and lgi1-3’R, 5’-CTTAGTGATCGAAGTCGAGATC-3’; lgi2, lg2-3’F, 5’-GGAA-GCTTGGCCAGGGGC-3’ and lg2-3’R, 5’-TCAAATCTCGGCAACTGG-3’.

Whole-mount in situ hybridization
The antisense lgi1 probe (same primers as above) was generated by using T7 RNA polymerase (Promega) using DIG RNA Labeling Mix (Roche). Whole-mount in situ hybridization was performed as previously described (Chuck et al., 2002) with the following modifications. Immature leaves were dissected and fixed in 3.7% formaldehyde, 5% acetic acid, 90% ethanol (FAA) (+ 1% Triton X-100, 1% DMSO) for 2 hours with rotation at room temperature. Pre-hybridization was carried out overnight at 55°C before adding the probes to the hybridization solution (6 × SSC, 50% ethanol (FAA) (+ 1% Triton X-100, 1% DMSO) for 2 hours with rotation at room temperature. Pre-hybridization was carried out overnight at 55°C before adding the probes to the hybridization solution (6 × SSC, 50% ethanol (FAA) (+ 1% Triton X-100, 1% DMSO) for 2 hours with rotation at room temperature. Pre-hybridization was carried out overnight at 55°C before adding the probes to the hybridization solution (6 × SSC, 50% ethanol (FAA) (+ 1% Triton X-100, 1% DMSO) for 2 hours with rotation at room temperature.
RESULTS

The dominant Lgn-R mutation affects leaf patterning and inflorescence development

Lgn-R was isolated as an M1 half-plant chimera following EMS mutagenesis of pollen from the B73 inbred background. In an EMS-induced chimera, half the plant carries the mutation whereas the other half does not (Candela and Hake, 2008; Neuffer et al., 2009). One half of the Lgn-R chimera appeared narrow with a missing ligule region whereas the other half of the leaf had expanded to near normal width and had some auricle tissue (Fig. 1B, arrowhead). The sector of mutant tissue included one entire half of a leaf and the margin of the other half. The mutation in the half-plant chimera was introgressed into different maize inbred backgrounds for further characterization. Analyses presented here were carried out in the B73 inbred background unless otherwise specified. Lgn-R heterozygotes are shorter in height and display reduction in leaf width and length (Fig. 2A-C; Table 1). Reproductive development is also affected in the mutant. The tassel, which is the terminal inflorescence, has fewer branches (Fig. 2D; Table 1), and the ears, which are inflorescence branches from the main shoot, frequently fail to form. The phenotype is more severe as a homozygote; the height is <30 cm and plants fail to produce any reproductive organs (Fig. 2A).

Blade morphology is moderately affected in Lgn-R whereas the sheath is relatively unaffected. Supernumerary epidermal hairs appear on the adaxial surface of the blade and, occasionally, ectopic abaxial hairs occur (Fig. 2E,F). The normal pattern of vasculature, however, suggests that the ectopic abaxial hair is not due to adaxial-abaxial polarity defects. The epidermal cellular morphology of the blade (Fig. 2G,J) and sheath (Fig. 2H,K) appears similar in wild type and Lgn-R. The most significant defect of Lgn-R leaves is found at the blade-sheath boundary, where auricles and ligule normally develop. In Lgn-R, these tissues are greatly reduced, with just a patch of ligule near the midrib (Fig. 2B,C). At the margins of Lgn-R leaves, sheath tissue extends into the blade or blade into sheath (supplementary material Fig. S1). Isolated patches of auricle cells are found intermixed between sheath cells (Fig. 2L) compared with the well-defined area of auricle cells in wild-type leaves (Fig. 2I). These results suggest that lgn might function in establishment of the blade-sheath boundary during leaf development.

The preligule band is not elaborated in Lgn-R

Preligular development, which occurs very early in leaf development, leads to differentiation of the ligule and auricle. In leaves that are the fifth to seventh from the meristem (P5-7), a horizontal band of small cells derived by both longitudinal and transverse anticlinal divisions appears along the epidermal surface (Fig. 3A-C). Following these rapid anticlinal divisions, periclinal divisions initiate and generate a ridge of cells growing out from the surface of the leaf, which will eventually form the ligule. We discovered that this process can be imaged by localizing the auxin efflux transporter, maize PINFORMED 1a (ZmPIN1a) by a YFP fusion (Carraro et al., 2006; Gallavotti et al., 2008; Gälweiler et al., 1998). As expected, ZmPIN1a was found expressed at high levels in lateral and intermediate veins (Fig. 3D) and the subcellular localization was towards the basal membrane of the cell. ZmPIN1a was also observed in the epidermal cells forming the preligule band (Fig. 3D-G). Interestingly, ZmPIN1a in these cells showed polarized localization towards the lateral membranes, suggesting a flow of auxin along the medial-lateral axis within the developing preligule band (supplementary material Fig. S2). The position of the preligular band coincides with the zone of intermediate vein anastomosis (Fig. 3E, arrowhead), indicating that an earlier signal exists to mark the position where the blade-sheath boundary will form.

Lgn-R leaves showed defects starting at P5. Small epidermal cells resulting from anticlinal divisions were observed, indicating that cells had initiated divisions (Fig. 3H,I). The small cells,
Table 1. Measurement of Lgn-R phenotype in B73 and W22

<table>
<thead>
<tr>
<th></th>
<th>Width (cm)</th>
<th>Length (cm)</th>
<th>Height (cm)</th>
<th>Tassel branch*</th>
</tr>
</thead>
<tbody>
<tr>
<td>B73</td>
<td>8.29±0.48</td>
<td>65.14±3.44</td>
<td>169.40±2.51</td>
<td>10.00±1.22</td>
</tr>
<tr>
<td>Lgn-R/+</td>
<td>3.57±0.61</td>
<td>38.42±4.47</td>
<td>115.25±16.26</td>
<td>5.60±0.89</td>
</tr>
<tr>
<td>W22</td>
<td>11.92±1.78</td>
<td>64.4±1.75</td>
<td>115.20±4.55</td>
<td>14.33±4.17</td>
</tr>
<tr>
<td>Lgn-R/+</td>
<td>6.02±0.99</td>
<td>46.6±1.71</td>
<td>91.80±10.21</td>
<td>5.37±0.89</td>
</tr>
</tbody>
</table>

*Number of lateral branches was counted. 

However, never formed a continuous band, but rather appeared in an erratic manner. Later-stage leaves showed some periclinal divisions that resulted in outgrowth of ligular patches near the midrib, but a continuous preligular band was never observed (Fig. 3J). Similarly, epidermal ZmPIN1a localization was found in some cells but not in a continuous band (Fig. 3K, L). The localization appeared only near the midrib where the ligular patches develop. These results suggest that the defect observed in Lgn-R begins prior to preligular band formation, at an early stage of leaf development when the boundary zone forms.

**Lgn-R and the liguleless mutants function in the same pathway**

In order to determine the effect of the Lgn-R mutation on lgl and lgs2 expression, different domains were harvested from developing leaves: the preligular band of P5-7 leaves, immature blade of P5-7 leaves and the shoot apex including P0-4 leaves. Our qRT-PCR analysis on these dissected tissues agrees with the previous studies (Moreno et al., 1997; Walsh et al., 1998) showing that in normal leaves, lgl1 accumulation occurs mainly at the preligular band region, whereas lgs2 has a broader expression pattern throughout the developing leaf (Fig. 4A). In Lgn-R leaves, both lgl1 (P=0.046) and lgs2 (P=0.026) mRNA accumulation levels were decreased at the preligular domain (Fig. 4A). lgs2 mRNA levels were also significantly decreased in the shoot apex (P=0.0091) of Lgn-R/+.

Previous attempts to localize the lgl1 mRNA on sectioned material were not successful (Moreno et al., 1997). Thus, considering the restricted domain of expression, we carried out whole-mount in situ hybridization. lgl1 was expressed in a narrow band near the base of an immature normal leaf and was absent in the lgl1-R mutant (Fig. 4B), which is consistent with the qRT-PCR analysis. In Lgn-R, lgl1 expression was found only near the midrib where patches of ligules form, suggesting that lgl1 expression correlates with the occurrence of the ligule (Fig. 4B).

lgl1-R mutant leaves lack all ligule and auricle, but the blade-sheath boundary is well defined (Fig. 4C), unlike Lgn-R, which lacks a sharp boundary at the margin of the leaf (Fig. 2B, C). In order to understand the genetic interaction between lgl1-R and Lgn-R, a double mutant was generated. As lgl1-R was in a W23 inbred background, the subsequent analysis was carried out with Lgn-R introgressed into W23. Lgn-R heterozygotes show a milder phenotype in W23 compared with that in B73; the ligule occasionally extends completely to the margin and auricles are reduced rather than absent (Fig. 4C). The plants, however, are still short and the leaves are shorter and narrower. Addition of the lgl1-R mutation to Lgn-R enhances the boundary defect of Lgn-R such that the ligule/auricle and the blade-sheath boundary fail to form altogether (Fig. 4C). This phenotype is more severe than each single mutant, suggesting a synergistic interaction between the two mutations. At the whole-leaf level, the phenotype was also synergistic, leading to a shorter and narrower leaf.

Double mutants were also made for Lgn-R and lgs2-R in a mixed genetic background. In lgs2-R mutants, ligule and auricle are found only at the margins of the leaf with a complete lack of blade sheath boundary over the midrib (Fig. 4D). In the double mutant, leaves are narrower, the midrib remnant of ligule always found in Lgn-R.

Fig. 3. Preligule development is defective in Lgn-R/+.

All of the leaves in this figure are presented with midrib towards the right. 

(A-C) Preligular band formation in P5 (A), P6 (B) and P7 (C) leaves of wild-type maize. Anticlinal divisions result in a band of small cells.

(D, E) ZmPIN1a localization (yellow, YFP) is observed at the preligular band of wild-type P6 (D) and P7 (E) leaves. The preligular band is positioned just below the anastomosis zone (arrowhead in E), where the intermediate (in) veins in between the lateral (la) veins merge. 

(F, G) Epidermal localization of ZmPIN1a of wild-type P6 (F) and P7 (G) leaves. The color index displays the relative brightness of the signal. Notice that the ZmPIN1a subcellular localization is polarized towards the lateral membranes.

(H-J) In Lgn-R/+ cells, divisions occur in P5 (H) and P6 (I) leaves but never form a continuous band. Yellow arrowheads mark some examples of aberrant divisions. P7 (J) leaves show preligule cells developing near the midrib.

(K, L) ZmPIN1a localization in P6 (K) and P7 (L) Lgn-R/+ leaves. ZmPIN1a appears in the preligule cells developing near the midrib of Lgn-R/+.
Patterning of the maize leaf

We looked for independently isolated alleles to confirm that the kinase is encoded by lgn. An insertion of the Activator (Ac) element was found in a collection of insertion lines generated by transposition of Ac from the waxy-m7(Ac) mutable allele (Cowperthwaite et al., 2002). Ac is an autonomous transposon and preferentially transposes to linked sites in the genome (Kunze et al., 1987; McClintock, 1951). The Ac insertion of tac905.29 and an 8-bp duplication resides in the 5′UTR of the kinase, −188 bp upstream of the predicted translation initiation site, and downstream of an in-frame AUG that is transcribed (Fig. 5A). GRMZM2G134382 expression was absent in tac905.29 (Fig. 5C); however, homozygous tac905.29 plants have no mutant phenotype (Fig. 5D, left).

In order to isolate additional alleles through germlinal transposition events of Ac, tac905.29 was outcrossed, self-pollinated and analyzed in the next generation. A few of the plants displayed a weak phenotype similar to the heterozygous Lgn-R, with narrow leaves and reduced auricles (Fig. 5D, middle). Through PCR-based genotyping and sequencing, those plants were determined to be heterozygous for the Ac insertion and carry a new allele resulting from Ac excision, referred to as Lgn-dAc. The excision site retained the 8-bp duplication with a 2-bp sequence difference (Fig. 5A). This Ac footprint results in a frameshift that would truncate translation products that initiate from the upstream AUG. Additional outcrosses to B73 and self-pollination produced plants that were homozygous for Lgn-dAc. Expression levels were restored to that of wild type (Fig. 5C). Leaves of these plants showed abnormal ligule structures (Fig. 5D, right) and the overall phenotype resembled homozygous Lgn-R, with plants failing to thrive.

The lgn gene consists of six exons that are predicted to encode a 414 amino acid membrane-associated kinase (Fig. 5A,B). The protein carries a 24 amino acid transmembrane domain and the kinase domain. Along with the kinase domain, LGN contains all 11 subdomains of a typical protein kinase. The invariant amino acids that define the catalytic domain are also conserved (Fig. 5B).

The Ala-to-Thr (A138T) substitution in lgn causes a frameshift that would truncate translation products that initiate from the upstream AUG. In Lgn-dAc, expression levels were restored to that of wild type (Fig. 5C). Leaves of these plants showed abnormal ligule structures (Fig. 5D, right) and the overall phenotype resembles homozygous Lgn-R, with plants failing to thrive.

**LGN maps to a grass-specific kinase**

A mapping population of 2800 chromosomes was used to place lgn in a 0.1 cM interval on maize chromosomal bin 9.03. This interval corresponds to 12 Mb of the genome, suggesting that recombination is greatly suppressed in the region owing to proximity to the centromere. Among the 80 genes predicted in this interval, we sequenced cDNAs of the likely candidate genes and found a lesion in a predicted serine/threonine kinase (GRMZM2G134382). Consistent with lesions induced by EMS, we found a G-to-A transition mutation in Lgn-R leading to a missense substitution of alanine to threonine at the amino acid level (Fig. 5A,B). In addition, whole-genome sequencing of a pool of 700 Lgn-R plants revealed no additional candidate mutations in the mapped interval (see Materials and methods). This kinase was broadly expressed, including shoot apices, tassels, ears and different domains of immature leaves (supplementary material Fig. S3), consistent with the pleiotropic defects observed during development. Expression remained unchanged in the Lgn-R mutants (supplementary material Fig. S3).

**Paralog expression is upregulated in Lgn-R**

A paralog of the lgn gene was found on chromosome 5 (GRMZM2G009506) and named sister of liguleless narrow (sln). LGN and SLN are 69.4% identical at the protein level. Orthologs for both LGN and SLN were not found in eudicots or monocots outside of the grass clade, indicating that these are likely to be grass-specific proteins (Fig. 5B). Phylogenetic analysis of the LGN homologs in maize, rice, Sorghum and Brachypodium showed that LGN was more closely related to its orthologs in other grasses than to any of the SLN orthologs (Fig. 5F). This result indicates that the gene duplication event occurred before speciation among these grasses.
sln mRNA was barely detectable in leaf tissues of wild type and tac905.29 homozygotes. However, it was greatly increased in all leaf subdomains of Lgn-R heterozygotes and in Lgn-dAc homozygous plants (Fig. 5G). Thus, the expression of sln appears to be an important and unifying component of the Lgn phenotype.

**DISCUSSION**

The semi-dominant Lgn-R mutant displays narrower and shorter leaves with a defective ligule and auricle. Lgn-R heterozygotes also have fewer tassel branches and some lack ears altogether, although phenotypic severity of the heterozygote varies with inbred. Defects are more severe in homozygotes, as the plants fail to proceed to reproductive development. The ligule defect initiates early in leaf development with reduced lg1 and lg2 expression and altered ZmPIN1a expression. lgn maps to a grass-specific kinase. Although an Ac insertion in the 5’UTR of the gene has no obvious phenotype, an Ac excision derivative that leaves a footprint has a Lgn phenotype. In further support of the kinase as the gene, kinase activity is reduced in Lgn-R mutants and expression of its paralog, sln, is greatly increased in both Lgn-R and Lgn-dAc.

**The nature of the Lgn mutation**

The Lgn-R mutation leads to a decrease in its kinase activity. If LGN was the only component in this signal transduction pathway, the tac905.29 homozygote might also show a similar phenotype owing to the absence of protein. However, the plants appear normal, suggesting that additional factors exist in the pathway to compensate for the absence of lgn in the tac905.29 allele. By contrast, the phosphorylation-defective protein encoded by Lgn-R might poison a complex that includes SLN or a common partner of both LGN and SLN. A similar result is seen in clv1 mutants, in which dominant-negative alleles of a gene have a more severe phenotype compared with null alleles (Diévart et al., 2003). How the footprint in the 5’UTR of the Lgn-dAc allele leads to a phenotype is unknown. Mutations have been documented due to changes in 5’UTR sequences that affect gene regulation (Pippucci et al., 2011; Wethmar et al., 2010); however, lgn transcript levels are not changed in Lgn-dAc. Another alternative is that translation begins from the upstream AUG. If this were the case, then the footprint results in a truncated polypeptide. Either this short polypeptide interferes with normal LGN function or its presence leads to translational skipping of the predicted AUG (Chua et al., 2012). Regardless of the mechanism, the increase in sln expression
in both Lgn mutants suggests a crucial role for functional LGN in negative feedback regulation of sln expression. Failure to block the accumulation of sln might be at the root of the Lgn defect, leading to the pleiotropic defects throughout development. If this model is correct, then a loss of function sln mutant would correct the Lgn-R and Lgn-dAc defects.

Revisiting the ligule pathway
Ligule development occurs early in maize leaf development (Fig. 6A). An inductive signal (the ‘preligule signal’) is thought to propagate from the midrib to the margin to initiate a localized increase in longitudinal and transverse anticlinal divisions in competent cells (Becraft and Freeling, 1991; Foster et al., 2004; Harper and Freeling, 1996). These divisions progress towards the margins of the leaf, forming a band of small cells called the preligular band, which separates the cells that will differentiate into blade from those that will differentiate into sheath (Fig. 3A-C) (Fowler and Freeling, 1996). The signal perception and propagation needed to establish the boundary zone and initiate a ligule involve LG1 and LG2 (Becraft and Freeling, 1991; Foster et al., 2004; Sylvester et al., 1990; Walsh et al., 1998). Both lg1 and lg2 transcripts are reduced in Lgn-R mutants, and double mutants of Lgn-R with either lg1-R or lg2-R are synergistic (Fig. 4). We hypothesize that LGN and SLN function as signaling components in this pathway.

RNA expression as well as mutant phenotypes suggest that LG2 and LGN function early in establishing the position of the ligule. lg2 mutants lack a blade-sheath boundary at the midrib, but the margins recover (Fig. 6B). Preiligule cell divisions do not occur at the midrib region (Walsh et al., 1998), indicating that neither is the boundary established in these cells nor are cells receptive to a signal. The ligules at the margin suggest that other factors are responsible for this recovery. Indeed, all ligule and auricle disappear in a lg1-R lg2-R double mutant (Harper and Freeling, 1996). The displaced ligules at the margins support an early role for LG2 in coordinating the boundary position from the center of the leaf outward. Lgn-R has a functional ligule over the midrib, indicating proper boundary initiation (Fig. 6C). The lack of distinct blade-sheath boundary towards the margins suggests that LGN functions in perceiving or transmitting the signal for boundary establishment outside of the midrib region. LG1 is thought to function later in the pathway during preiligule cell division. Transverse divisions of the preiligule band occur in lg1 mutants, but longitudinal and periclinal divisions do not (Fig. 6D) (Becraft et al., 1990). The lack of any blade-sheath boundary in lg1-R Lgn-R double mutants, however, suggests that LG1 might also play a redundant role with LGN in establishing the position of the ligule.

In wild-type leaves, development of the preiligule band is coincident with subcellular localization of the auxin transporter ZmPIN1a (Fig. 3D-G). The preiligule epidermal localization occurs at lateral membranes, which is perpendicular to that of vascular ZmPIN1a at the position of intermediate vein anastomosis. Early studies have shown that intermediate veins initiate from the tip of late P3 to early P4 leaves and differentiate basipetally (Sharman, 1942). At P7 to P8, the veins anastomose and only one intermediate vein enters the presumptive sheath. In Lgn-R leaves, epidermal ZmPIN1a is observed in patches of cells adjacent to the midvein in a discontinuous pattern (Fig. 3L). The localization expands into a short band of cells, which seems to initiate from the lateral vein towards the margin. These observations support the hypothesis that LGN plays a role in establishment of the boundary zone and that the defective allele has interfered with its normal function.

Previous models proposed that a preiligule signal initiates near the midrib and travels towards the leaf margins. Our data reveal that auxin and LGN are two new components in establishment of the ligule position and its propagation. Our data also suggest that the signal is discontinuous along the medial-lateral axis and forms from large veins, which become connected as signals meet in between the veins (Fig. 6A). How this flow of auxin integrates with lg1, lg2 and lgn in establishing the preiligule band requires further investigation.

Coordination of leaf width and preiligule establishment
The narrow leaf phenotype of Lgn-R suggests the involvement of LGN in leaf expansion. Previous studies of maize mutants with narrow leaves suggest that different domains exist along the medial-lateral axis of the maize leaf. The leaves of narrow sheath (ns) mutants are half the width of normal siblings but have normal ligule and auricles (Scanlon et al., 1996). The ns defect removes the marginal domain of the leaf, resulting in a blunt-edged leaf (Scanlon et al., 1996; Scanlon and Freeling, 1997). The dominant mutant Wavy auricle in blade1 (Wab1) also has narrow leaves, but its leaf margins are tapered, as in wild type, and double mutants with ns are additive (Hay and Hake, 2004). Wab1 homozygotes lack a ligule in a domain internal to the marginal domain, suggesting that Wab1 has failed to elaborate or expand this domain. Similar to Wab1, Lgn-R leaves have a tapered margin, suggesting that the Lgn-R defect also occurs inside the marginal domain. In
addition, Wab1 is synergistic with lg1 (Foster et al., 2004). A difference between Wab1 and Lgn-R is that Wab1 overexpresses lg1 and recovers auricle and ligule at the very margin, whereas Lgn-R does the opposite. These comparisons suggest that signals required to establish the position of the ligule are coordinated with signals that operate internal to the marginal domains to promote leaf width expansion. Future studies with LGN will be aimed at identifying the signals involved in the cell-cell communication.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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