Division and differentiation during normal and liguleless-1 maize leaf development

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Summary

The maize leaf is composed of a blade and a sheath, which are separated at the ligular region by a ligule and an auricle. Mutants homozygous for the recessive liguleless-1 (lgl) allele exhibit loss of normal ligule and auricle. The cellular events associated with development of these structures in both normal and liguleless plants are investigated with respect to the timing of cell division and differentiation. A new method is used to assess orientation of anticlinal division planes during development and to determine a division index based on recent epidermal cross-wall deposition. A normal leaf follows three stages of development: first is a preligule stage, in which the primordium is undifferentiated and dividing throughout its length. This stage ends when a row of cells in the preligule region divides more rapidly in both transverse and longitudinal anticlinal planes. During the second stage, ligule and auricle form, blade grows more rapidly than sheath, divisions in the blade become exclusively transverse in orientation, and differentiation begins. The third stage is marked by rapid increase in sheath length. The leaf does not have a distinct basal meristem. Instead, cell divisions are gradually restricted to the base of the leaf with localized sites of increased division at the preligule region. Divisions are not localized to the base of the sheath until near the end of development. The liguleless-1 homozygote shows no alteration in this overall pattern of growth, but does show distinct alteration in the anticlinal division pattern in the preligule region. Two abnormal patterns are observed: either the increase in division rate at the preligule site is absent or it exhibits loss of all longitudinal divisions so that only transverse (or cell-file producing) divisions are present. This pattern is particularly apparent in developing adult leaves on older lgl plants, in which sporadic ligule vestiges form. From these and results previously published (Becraft et al. (1990) Dev. Biol. 14), we conclude that the information carried by the Lgl+ gene product acts earlier in development than formation of the ligule proper. We hypothesize that Lgl+ may be effective at the stage when the blade–sheath boundary is first determined.

Key words: maize, leaf development, division, lgl.

Introduction

Plant development is both repetitive and progressive in nature. These features are particularly evident during leaf development, when a leaf primordium produced by the shoot meristem undergoes an orderly pattern of growth while the next leaf primordium is just being initiated. At any given time, the plant body is initiating new growth as well as directing the progressive development of mature structures. Investigators have used several approaches to understand how these processes are coordinated. One approach relies on histological sections to examine division patterns and gain information about morphogenesis on a cellular level (e.g. Lyndon, 1982; Jeune, 1984; Sharman, 1942). Another approach is to investigate the fate of individual cells in the shoot meristem by analyzing genetic mosaics (Stewart and Derman, 1979; Poethig and Sussex, 1985; Poethig, 1984). A third approach that helps to clarify when and how developmental information is expressed is to investigate leaf developmental mutants. (Freeling and Hake, 1985; Freeling et al. 1988). Combined, these three approaches could provide a comprehensive view of what controls patterns of leaf development.

The maize leaf is a useful system to combine these approaches to investigate patterns of leaf development. A normal maize leaf consists of a blade separated from a sheath by a ligular region, which consists of an auricle and a ligule. The wedge-shaped auricle is composed of cells distinct in size and appearance from both blade and sheath cells. The ligule is a row of epidermal cells that project from the base of the auricle on the adaxial leaf surface. Together the ligule and auricle contribute to leaf attitude; at their junction the blade extends at 45° away from the sheath, which clasps the vertical axis of the plant. This ligular region is particularly informative for study because it is morphologically simple, its developmental history is accessible, and there are a number of mutations specific to it (Hake et al. 1985; Freeling et al. 1988; Becraft et al. 1990). For these
reasons, research has focused on understanding what controls the position and timing of ligule development in maize plants.

Based on histological study, the ligular region is first visible when the epidermis and underlying mesophyll cells divide in a periclinal direction (Sharman, 1942; Hake et al. 1985; Becraft et al. 1990). These events occur at plastochron 4 or 5 (using the terminology of Sharman, 1942), which is well after the leaf primordium has emerged from the apex and is less than 1 cm long. The midvein, lateral, and intermediate veins have differentiated in the primordium at this time, but the blade is not yet distinct from the sheath. Ligule differentiation progresses from the central region of the leaf near the mid-rib toward the margin (Hake et al. 1985). The sequence of auricle formation is less clear but appears to be closely coordinated temporally and spatially with the production of the ligule (Becraft et al. 1990).

Mutants that alter maize leaf development show a remarkable diversity of effect. Although the overall strap-shape of the leaf varies in only a few mutants (such as in the various dwarf mutations), there are many mutations that alter developmental timing or positioning of the ligular region (Freeling et al. 1988). Several known gene products contribute to normal placement and differentiation of this ligular region in maize leaves. One of these, the liguleless-1 (lgl) gene, appears to be particularly useful for understanding how the timing and positioning of leaf structures are coordinated. Becraft and coworkers (in press) initiated an investigation into the recessive lgl mutant. They showed that in plants homozygous for liguleless-1 (mapped to chromosome 2s; see Emerson et al. 1912), the blade–sheath boundary is less well defined than in normal plants and, when fully expressed, the ligule and auricle are absent entirely. Based on genetic mosaic analysis of clonal sectors of liguleless tissue within normal, they proposed that the Lg-1 gene is autonomous in both epidermal and internal tissues, but with different functions in these tissues. They proposed that this gene product is part of a communication scheme that may include several other ligule-specific mutants.

A great deal of general information about how monocot leaves grow is already known, but there is little detailed information available about the pattern of division, cell extension and differentiation during ligule development. Clonal analyses confirmed the work of Sharman (1942) that the maize leaf is derived from the outer two layers of the meristem (Poethig, 1984; McDaniel and Poethig, 1988). In addition, Poethig (1984) showed that these cell layers are not strictly fated, but that cells from the outermost layer may contribute to either epidermal or mesophyll cells. Furthermore, the position of cells at the site of primordium initiation at the apex appears to be more important in determining final leaf structure than the layer from which they were derived (Poethig, 1984).

The general basipetal growth pattern of the monocot leaf has been deduced from histological description (Sharman, 1942; Kaufman, 1959) and by measuring relative growth zones (Kemp, 1980; Davidson and Milthorpe, 1966). The growing zone has been thought to be restricted to the base of the leaf. Both division and cell extension contribute to overall growth, but few of these studies distinguish division from extension, despite the fact that they often occur separately. Mitotic indices (Cusset, 1986; Poethig and Sussex, 1985), autoradiography, and estimates of increase in cell number over time (Maksymowych, 1973) have been used to locate division sites in dicots. These techniques have not been applied to monocots, probably because very young developing leaves are surrounded by older leaf sheaths and are therefore inaccessible. Poethig (1984) showed by clonal analysis that intercalary and not exclusively marginal cell divisions direct blade expansion, that divisions cease first at the tip, and that cells are polarized with respect to the long axis of the maize leaf.

In this paper, we attempt to further clarify the role of Lgl+ during normal leaf and ligule development. Proper interpretation of mutants depends on a detailed description of the duration and rate of cell division relative to cell expansion and differentiation. Such baseline information is lacking for the maize leaf. For this reason, we have used a new method to determine where and when division, extension and differentiation occur on the adaxial leaf surface throughout development. The wild-type pattern of growth is then compared with the pattern observed in liguleless-1. Comparisons between the wild-type and homozygous mutant allow us to speculate on the site of action of the lgl mutation and provide information about the order of morphogenetic events during development of the ligular region in maize.

Materials and methods

Plants used for this study were of standard inbred line W23 or lgl homozygotes in a W23 background (lgl gl2; et stock was backcrossed 6 times to W23) and was provided by R. Scott Poethig (University of Pennsylvania). Seeds were grown in greenhouses on the campus of the University of California, Berkeley and were harvested for study at 2 different stages of growth, arbitrarily defined as ‘young’ and ‘old’. Young plants were designated as those in which leaf 1 (as counted from the bottom of the plant) was not yet fully senescent and leaves 5 and 6 were just emerging from the enclosing sheaths. Upon dissection, these ‘young’ plants had a total of 8–10 leaves. Old plants were designated as those in which the bottom embryonic leaves were senescent and leaves 7 or 8 were just emerging from the surrounding sheaths. Upon dissection, these ‘old’ plants had a total of 12 to 14 leaves and the apical meristem had begun to differentiate a tassel primordium. Each leaf was given both a plastochron (P) and a leaf (L) number. Following the terminology of Sharman (1942), the plastochron number defines the age of the leaf primordium in terms of its distance from the apical meristem: P1 is the youngest leaf primordium and closest to the apex, P2 is the next youngest primordium, and so forth. Leaf number, on the other hand, is counted from the base of the plant toward the apex and defines the position of the leaf relative to the developmental stage of the plant. A lower numbered leaf is produced during the juvenile stage of development, a higher
number leaf is produced during the adult stage. Here we describe each leaf by both plastochron and leaf number in order to designate both leaf age and plant stage simultaneously. For example, L8/P2 is a small primordium, second in position from the apex, and produced during the adult stage of development, whereas L3/P2 is the same size primordium, also second in position from the apex, but produced earlier during the juvenile stage. Because there is inherent variability in developmental timing among plants (due to genetic, environmental or physiological factors), this numbering system permits only approximate comparisons, but also gives a more accurate developmental description of individual leaves.

Plants of given age and phenotype were dissected by removing each leaf at the base of the sheath, as close to its insertion at the node as possible. This was done by slicing with a fine knife and gently unrolling the leaf. Outer, more rigid sheaths were sliced longitudinally near the mid-region to facilitate removing the sheath and blade with minimal damage. Leaf dimensions were recorded and positions along the length of blade and sheath were chosen using an x-y coordinate system. The midvein was defined as the y axis and the ligule region the x axis. The length of the +y axis represented blade length and that of the –y axis represented sheath length. All positions on the leaf were then selected from the +x, +y and +x, –y quadrants. Three or four equally spaced positions along the y axis were chosen in each quadrant. The x position was chosen as equidistant from the blade or sheath margin and the y axis. This method insured that cells to be analyzed were equidistant between the midvein and margin.

At each designated position along the leaf, a cast of the adaxial leaf surface was prepared following a replica method of specimen preparation. This method preserves the shape of waxes, hairs and delicate structures and obviates shrinkage problems encountered in traditional methods of tissue fixation (Williams et al. 1987). Furthermore, as a nondestructive sampling technique, the method allows additional study of the same leaf structure that had been sampled for SEM (Sylvestre et al. 1989). In brief, a dental impression medium of low viscosity (Exaflex, Patterson Dental Supply, Sunnyvale CA) was applied to the surface and allowed to harden at room temp for 3–5 min. The surface replica was removed and nested into a support mold of high viscosity dental impression medium (Kerr-Reflect, Patterson Dental Supply, Sunnyvale CA). This second mold provided support in such a way that the replica could be filled with Spurr’s resin (Spurr, 1969) to make a positive cast of the leaf surface. The resin-filled replica was polymerized at 70°C for 24 h. The cast was removed from the replica nest, mounted on aluminum stubs with epoxy glue, coated with 30 nm gold from a Polaron sputter coater, and observed on an ISI DS-130 SEM equipped with a LaB₆ filament and operating at an accelerating voltage of 15 kV.

The viewing surface of the leaf cast was flat and maintained perpendicular to the scanning beam to prevent image distortion beyond the normal 3% inherent to the microscope. Each cast was photographed at the same magnification for quantitative analysis. Photographs were then enlarged 2× for ease of measurement and cell lengths were recorded. At each stage of development, presence or absence and location of the following surface features were recorded: waxes, hairs (including macro-hairs, prickle hairs, and bicellular micro-hairs) and stomata.

Evidence for recent epidermal divisions was determined by assessing the presence of cross-walls that appear shallow relative to neighboring cross-walls. Variations in cross-wall depth are assumed to correlate with relative time since cytokinesis. This is based on the observation that older walls are thicker and broader than younger walls because they contain more wall material and a thicker cuticle. Wall thickness differences are visible in sectioned material as well as from a surface view. The SEM permits such an evaluation because shadows and depth are highlighted by the 3-dimensional image (Fig. 1). Assessment of recent cross-wall formation was based on the following criteria: a single cross-wall was compared against both the walls it abuts at 90° as well as the surrounding two parallel walls. The cross-wall was considered recent if it was shallower and thinner than the aforementioned three neighboring walls. In cases where long packets of cells show a number of parallel cross-walls similar in depth, which occurs frequently in rapidly dividing tissue, the cross-walls were compared only against the perpendicular abutting wall. The presence of a recent cross-wall was considered evidence of a single division event and was therefore counted as such. A ‘division index’ was derived by counting the number of recent cross-walls in regions of equal size at each designated position on the adaxial leaf surface. The number of recent cross-walls was divided by the number of cells counted minus the number of recent cross-walls. This approach removed the artifactual counting of two cell divisions per one cross-wall. Because a mitotic index refers specifically to the ratio of unaphase figures in a field of cells, we have designated the term ‘division index’ to the ratio of recent cross-walls in a field of cells. The highly polarized cell divisions that are characteristic of maize leaves makes this approach straightforward: most divisions are either transverse (perpendicular to the long axis of the leaf and contributing to length) or longitudinal (parallel to the long axis of the leaf and contributing to width).

Results

Description of surface features of the blade and sheath

The adaxial surface of the mature maize leaf is distinguished by the polar orientation of its cells and the relatively few different types of cells (Fig. 1). During its determinate development, the maize plant undergoes a progressive aging from juvenile to adult. Overall plant form reflects these states. The five to six leaves present in the embryo prior to germination senesce early during maturation of the plant. These juvenile leaves are distinctive because they are narrow and short, lack differentiated hairs, and are coated with epicuticular wax (Fig. 1A). The mature adult leaves are long, broad and possess several different types of cells including long and short cells, bulliform cells, silica cells, interstomal cells and various hairs (Fig. 1D–F). Wall junctions are crenulated and interlocking when the cells are mature (Fig. 1D). Bulliform cells have distinct elongated ridges on their surface when not turgid (Fig. 1D). Presumably these ridges accommodate the extra expansion associated with increasing turgidity. Recent divisions are apparent where a cell wall is shallower relative to its neighboring walls (arrow, Fig. 1C). Three hair types form on the adaxial surface of the adult blade. These are macrohairs, prickle hairs and bicellular microhairs (Fig. 1D–F). The macrohairs emerge from a specialized group of basal cells overlying a row of bulliform cells (Fig. 1E). When the bulliform
and the hair's basal cells are fully turgid, the macrohair projects at 45° from the leaf surface toward the tip of the blade (Fig. 1E). When these cells lose turgidity, the macrohair rests parallel to the long axis and against the leaf surface (Fig. 1F). Prickle hairs are short wedge-shaped cells that recurve toward the blade tip, emerging from cells that lie adjacent to veins (arrow, Fig. 1F). Prickle hairs lack cross-walls or specialized basal cells. The third type of hair, bicellular microhairs, are found throughout the blade surface, also frequently adjacent...
to costal cells or bulliform cells (Fig. 1C and D). They emerge from a small cell, which is derived from an unequal division of an intercostal cell (Fig. 1C). The microhair divides once during development to produce two cells. The outer cell has a thin wall, contains a nucleus at its tip, and often appears collapsed by SEM and transparent by light microscopy. The basal cell is thick-walled, cone shaped and recurved toward the blade tip.

Stomata are distinctive in the mature blade (Fig. 1A and E). They are usually aligned with the long axis of the leaf in rows that are adjacent to intermediate and lateral veins. Some veins are adjacent to rows of bulliform cells rather than stomata. Interstomatal cells are long and their endwalls curve around the stoma. Mature blade stomata have two subsidiary cells on either side of the pore as well as two distinctive protrusions on either end of the pore.

Sheath cells (Fig. 1B) are distinct in morphology from blade cells (Fig. 1A, C, E and F). Sheath cells lack specialized walls (crenulations or ridges), lack hairs of any type on the adaxial surface, and are of two general sizes. Mature sheath cells are straight-walled and rectangular. Sheath stomata are smaller and broader than blade stomata and their lateral subsidiary cells are more broadly triangular. Although present in the sheath stomata, the bulbous protrusion on either end of the pore is less distinct than that in the blade.

**Description of the ligular region**

Previous studies showed that, in longitudinal section, the ligular region is first visible when a group of epidermal cells appear shorter in the longitudinal dimension than neighboring blade or sheath cells. These cells undergo a periclinal division, the underlying mesophyll cells enlarge and also divide periclinally (Becraft et al. 1990). A three-dimensional surface view shows that prior to this time there is a local increase in division rate combined with a decrease in cell extension, forming a band of small cells termed a preligule band (Fig. 2A). These cells grow out to form both the ligule and auricle. Ligule outgrowth follows a distinct pattern. First, a ligular ridge continuous with the sheath is derived from cells of the preligule band (Fig. 2B and G), which ultimately elongate in the adaxial direction and bend toward the blade tip. Eventually the ligule rests against the blade (Fig. 2E). A group of small cells derived from the preligule band are covered by the outgrowing ligule (Fig. 2G and H).

Although the preligule band forms evenly around the leaf surface, the initial events of ligule outgrowth occur at different rates (Figs 2C and D). Ligule outgrowth progresses from a lateral position near but not directly over the midvein out toward the margin and in toward the midvein. Therefore, the rate of ligule outgrowth is slower over the midvein and the immediately adjacent lateral veins (Fig. 2C). Eventually, ligule outgrowth equalizes around the leaf circumference so that the mature ligule is continuous and of even dimension from margin to margin (Fig. 2D).

The mature ligule emerges as an apparent extension of sheath tissue; that is, it usually projects from the thickened and rigid mature sheath and lies appressed against the blade (Fig. 2E). The cells of the ligule are smooth, elongate and tapering (Fig. 2E). Behind the ligule and in the blade is a wedge of small evenly spaced cells that compose the auricle. Removal of a young ligule reveals small evenly sized cells of the auricle grading into blade cells (Fig. 2F). Eventually these cells in the auricle elongate but after the ligule and much of the blade is mature. Auricle formation is more apparent on the abaxial surface where the distinct auricle cells form near the midrib and extend toward the leaf margin (not shown).

**Transition from the juvenile to the adult stage in W23 inbred line: young plant**

Figs 3–6 (corresponding to leaves 3–6 respectively) display the progression of development along the leaf surface and from leaf to leaf in an individual plant. Only the leaf number will be referred to here, because it is most relevant to the transition from juvenile to adult stages. Based on the distribution of epicuticular wax and hairs in sequential leaves, the juvenile to adult transition is gradual. The blade of leaf 3 lacks hairs entirely and is covered with wax throughout its length (Fig. 3A–C), whereas a waxy cuticle covers only the distal 2/3 of leaf 4 (Fig. 4A and B) and the distal 1/3 of leaf 5 (Fig. 5A). Leaf 6 lacks wax entirely and shows a characteristic sequence of hair development from above the ligule to the tip of the blade (Fig. 6A–D).

Cell differentiation follows a similar gradual transition from juvenile to adult stage. Between leaf 3 and leaf 6, the transition from juvenile to adult, and from immature (undifferentiated) to mature (differentiated) is apparent (Figs 3–6). The ligule acts as a boundary between two patterns of division and differentiation: one unique to the blade and the second unique to the sheath. Leaf 3 is a fully mature juvenile leaf: division has ceased in the blade and sheath as has differentiation of mature specialized cells (Fig. 3). Leaf 6 is an immature adult leaf: division is still occurring in the bottom half of the blade (Fig. 6D) and along the entire sheath (Fig. 6E). The blade has begun to differentiate hairs and stomata whereas the sheath has not yet begun to differentiate stomata (Fig. 6E). These observations indicate that during this transition stage, sheath and blade have their own agenda of development with blade differentiating before the sheath.

**Localized division and ligule formation during development of W23 inbred line: old plant**

Figs 7–10 (corresponding to plastochrons 5–2, respectively) show the pattern of division and differentiation associated with ligule formation in an old plant. For this section, the plastochron number will be used exclusively because it is more pertinent to the pattern of ligule growth. Plastochron 2 has the distinctive shape of an open-sided cone (Fig. 10A). The leaf primordium emerges from around the circumference of the apex, fans out slightly immediately above the insertion point, and tapers towards the leaf top but does not completely...
Fig. 2. Ligule formation in wild-type W23 plant. (A) Preligule division band. The junction between blade and sheath is separated by packets of small cells derived from both longitudinal and transverse divisions (t=recent transverse division; l=recent longitudinal division). (B) Forming ligule ridge. The small cell packets from A contribute to both ligule and auricle growth. (C) Ligule outgrowth is initially slower over the mid-rib, where the ridge is less pronounced (left of photograph). (D) Eventually ligule outgrowth is equalized so that the ligule forms a continuous ridge around the adaxial surface. (E) Ligule fringe. (F) Removal of the ligule fringe reveals small cells that contribute to auricle formation (b=blade; sh=sheath; l=ligule; arrow=small cells underlying the removed ligule). (G) and (H) Two sequential stages in ligule outgrowth showing small cells behind the ligule (G) that eventually are covered as part of the auricle (H). Scale bar, (A, G, H), 50 µm; (B), 65 µm; (C), 20 µm; (D), 120 µm; (E, F), 100 µm.
Figs 3–6. Transition from juvenile to adult and pattern of differentiation at designated positions on the adaxial leaf surface in sequential leaves on a W23 plant. For this and all subsequent figures, each column of micrographs corresponds to selected positions along an individual leaf. The leaves are oriented with the blade tip at the top of the page (A), the ligular region in the third of fourth position (C or D) and the sheath base in the last position at the bottom of the page (D or E).

Fig. 3. Leaf 3/Plastochron 10: a mature juvenile leaf with waxes on the entire blade surface (A–C). The sheath lacks wax.

Fig. 4. Leaf 4/Plastochron 9: a juvenile to adult transition leaf with waxes restricted to the upper 2/3 of the blade (A–B). The ligule fringe is not shown, but is present between C and D.

Fig. 5. Leaf 5/Plastochron 8: a juvenile to adult transition leaf with waxes restricted to the upper third of the blade (A) and hairs differentiating above the ligule (B–C). Cells are dividing in the lower 1/2 of the blade only.

Fig. 6. Leaf 6/Plastochron 7: an immature adult leaf. The ligule ridge is barely evident (base of D). Waxes are absent. Cells are still dividing near the bottom of the leaf blade (B–C). Scale bar, 90 μm. All micrographs in Figs 3–6 are at the same magnification.
Figs 7–10. Pattern of ligule formation at designated positions on the adaxial leaf surface in sequential leaves on a W23 plant.

Fig. 7. Leaf 8/Plastochron 5: immature adult leaf with forming ligule ridge (C). Hair differentiation is restricted to the upper 2/3 of the blade (A and B). Cells are dividing on the lower 2/3 of the blade (B and C).

Fig. 8. Leaf 9/Plastochron 4: immature adult leaf. The ligule ridge is less pronounced (C). Hair differentiation has begun on the upper 1/3 of the blade, where terminal divisions are visible (A). Cells are dividing throughout the leaf.

Fig. 9. Leaf 10/Plastochron 3: immature adult leaf. The leaf is cone-shaped and wrapped around the apex (A). Blade cells (B) and sheath cells (D) are distinct (compare with Fig. 10B) and increased division activity is evident in the preligule band (C).

Fig. 10. Leaf 11/Plastochron 2. The young leaf primordium (A) has not yet enclosed the apex. Cells have not yet differentiated (B). A bend in the course of the cell files and veins (arrows, B) indicates where the blade–sheath border will be but a preligule division band is not yet apparent. Scale bar, 45 μm. All micrographs are at the same magnification except 9A (1 cm=200 μm); 10A (1 cm=60 μm) and 10B (1 cm=70 μm).
surround the apex. The cell files and veins bend at the junction between the narrow and the broader portion of the base (arrows; Fig. 10B). Differentiation of blade versus sheath cells is not yet evident at this stage.

The growing leaf edges overlap and cover the apex, as seen in the next leaf up from the apex (P3; Fig. 9A). When unfurled, the leaf is ovate with its widest portion one quarter of the leaf distance up from the base. Blade cells (Fig. 9B) have begun to differentiate from sheath cells at this stage (Fig. 9D) and the preligule band of rapidly dividing cells is visible near the leaf base (Fig. 9C). In addition to dividing more frequently, cells in this region are not elongating after cytokinesis. This conclusion is based on a comparison of cell dimension to the number of new cell walls.

There is a change in division orientation in the preligule band as well as a change in division rate. Divisions in the sheath are primarily transverse in orientation producing long relatively uniform cell files (Fig. 9D). The preligule band has increasing numbers of longitudinal divisions (Fig. 9C). Divisions in the blade are also primarily transverse with increasing numbers in the longitudinal dimension, which correlates with the local increase in leaf width (Fig. 9B). Meanwhile, epidermal and subepidermal cells divide periclinally to initiate the outgrowth of the ligule (as described by Becraft et al. 1990). Ligule outgrowth is first apparent by plastochron 4 and 5 as a ridge across the adaxial surface (Fig. 8C and 7C). Subsequently, the ligule grows away from the blade surface (as in Fig. 6D), where the ligule ridge is barely visible at the bottom of the photograph, and Fig. 5D). Eventually the ligule appears as a fringe of cells resting against the blade surface (as in Fig. 3C).

Prior to ligule formation (at P2), the entire leaf surface is dividing anticlinally in both transverse and longitudinal orientation (Fig. 10B; see also Fig. 19A). A localized increase in numbers of anticlinal divisions in the ligular region during P3 and P4 predicts the site of the periclinal divisions that produce the ligule and auricle (Figs 8C and 9C). The time of cell differentiation correlates with these anticlinal divisions; i.e. blade cells are distinct from sheath cells and hair differentiation begins near the blade tip just as the ligule is forming (Fig. 8A). Note that all divisions near the tip of the young blade are final predifferentiation divisions: that is, they are all unequal and in the process of forming either hairs or stomata (Fig. 8A). Ligule outgrowth becomes more apparent at the same time that the upper third of the blade completes differentiation (P5; Fig. 7A). Divisions continue throughout the length of the sheath but terminal differentiation is not yet apparent morphologically.

Transition from juvenile to adult stage during development of a liguleless-1 homozygote (lg1/lg1): young plant

The overall morphology and histology of the lg1 homozygote has been described elsewhere (Becraft et al. 1990). The results presented here examine the sequential development of lg1/lg1 plants from the standpoint of division, extension and differentiation. Macroscopically, the mutation removes the ligule and auricle so that the leaves show an upright habit. However, the blade and sheath are still distinctive so the region joining the two, the presumptive ligular region, can be examined for cell type and stage.

Juvenile and immature adult leaves from a young lg1 plant are shown in Figs 11–14 (corresponding to leaves 3–6 respectively). Leaf number will be used exclusively in this section. Development is similar to that of a normal plant with the exception of events occurring at the ligular region. That is, just as in the wild-type plant, the juvenile waxes in the liguleless plant are gradually replaced over at least two leaves by adult hairs (Figs 11–13). Also, the basipetal direction of cell differentiation is similar to that in wild-type: hairs and stomata differentiate in the blade before stomata in the sheath.

The liguleless and wild-type plants differ in the pattern of division and elongation in the ligular region. A mature juvenile leaf of a homozygous mutant (Fig. 11) lacks a ligule and auricle. Instead, there is gradual change in the ligular region from blade cells covered with wax to sheath cells free of wax (Fig. 11C–E). Individual cells at the border between sheath and blade show partial wax production (Fig. 11D). These observations suggest some cells in the ligule region of liguleless leaves are intermediate in their expression of blade versus sheath features.

Usually a preligule band of cells is not evident during development of a liguleless leaf (Fig. 13D). However, young leaves, such as leaf 6 (Fig. 14D), occasionally show a band of increased division activity in the presumed ligular regions. These divisions are exclusively transverse in orientation, compared with true preligule band divisions, which are both longitudinal and transverse (compare cells in Figs 7C, 8C, 9C with Fig. 14D).

Divisions and formation of ligule vestiges during development of liguleless-1 homozygote (lg1/lg1): old plant

The expression of the lg1 mutation is different during the adult stage from that during the juvenile stage of development. Adult lg1/lg1 leaves produce ligule vestiges in the vicinity of the presumptive ligular region (Figs 15B and C; note that the enlargement is different from the other micrographs). Ligule vestiges always appear at the boundary between blade and sheath cells but are often unevenly distributed across the leaf surface. Blade cells are normal in appearance in leaves that produce ligule vestiges (Figs 15A and 16A,B), as are sheath cells (Figs 15D and 16D). Ligule vestiges have a similar morphology to normal ligules in that they are appressed against the blade surface and are fringed, but they are short and often angled toward the midrib (Fig. 15C) or else inserted between two veins (Fig. 15B). Early stages in the formation of these ligule vestiges are visible in immature adult leaves (Fig. 16C). A preligule division band precedes the formation of ligule vestiges (Fig. 17C), but these rapidly dividing
cells are sporadically distributed around the leaf surface rather than being localized into a continuous band as in wild-type. Furthermore, the divisions that produce ligule vestiges are both transverse and longitudinal in orientation, as is characteristic of normal development. In the mutant, a short segment of a normal preligule band (arrow, Fig. 17C) is often adjacent to a short segment of abnormal tissue in which cell divisions are all transverse (arrowhead, Fig. 17C). It is likely that these normal but sporadic preligule bands will produce ligule vestiges during subsequent development of the leaf, whereas the abnormal (transverse-only) divisions correspond to regions where ligule will be absent.

Relative growth rate and differentiation of blade versus sheath in young and old plants of wild-type and liguleless phenotypes

Growth is quantified by comparing the time of divisions
Figs 11-14. Transition from juvenile to adult and pattern of differentiation at designated positions on the adaxial leaf surface in sequential leaves of lgl/lgl: a young plant with no ligule vestiges.

Fig. 11. Leaf 3/Plastochron 8: mature juvenile leaf with wax on entire blade surface (A-C). Ligule and auricle are absent but the blade–sheath border occupies the same position as wild-type and can be recognized microscopically by the gradation at D of waxy blade cells (as in A-C) to wax-free sheath cells (as in E).

Fig. 12. Leaf 4/Plastochron 7: juvenile to adult transition leaf. Waxes occupy the upper 2/3 of the blade as in wild-type (A-B). Sheath and blade cells are distinct and grade together at a normal position (between D and E).

Fig. 13. Leaf 5/Plastochron 6: a juvenile to adult transition leaf. Waxes occupy the upper 1/3 (A) and developing hairs occupy the lower 2/3 of the blade (B–C). There is no sign of ligule vestiges or the divisions that precede them.

Fig. 14. Leaf 6/Plastochron 5: an immature adult leaf lacking wax. A presumptive preligule division band with transverse-only type divisions is apparent but has formed at a later plastochron than usual (D). Scale bar, 90 μm. All micrographs are at the same magnification.

(Figs 19 and 21) with the change in leaf proportions and hair cell differentiation (Figs 20 and 22). A division index of sequential leaves from a single plant compares sites of increased division activity as well as orientation of the new divisions during development of a normal plant (Fig. 19). Initially, the leaf divides throughout its length with divisions exclusively transverse near the leaf base (Fig. 19A). Divisions are both transverse and longitudinal along the rest of the leaf surface. This division pattern corresponds with leaf shape: longitudinal divisions, which presumably contribute to leaf width, predominate in the widest portion whereas transverse divisions, which contribute to leaf length, predominate in the more narrow linear portion at the base.

Fig. 19 indicates several stages where anticalinal division activity peaks. One peak is near the base of a L10/P3 leaf, where divisions are locally more rapid than neighboring regions so that a band of small cells is produced (Fig. 19B). This site of activity is the preligule band, which predicts the location of the future ligule. The blade tip has ceased dividing at this stage. Divisions are both transverse and longitudinal at the site of the preligule band as well as immediately above the band in the blade, but are exclusively transverse near the blade tip. Cells in the now-recognizable sheath are derived from transverse divisions exclusively.

Another peak of division occurs in the next plastochron at the base of the blade above the developing ligule (Fig. 19C). Division has ceased in the upper third of the blade and is slow in the sheath at the time of ligule initiation. The few divisions present in the sheath at this time are exclusively transverse. Cell divisions in the blade are increasingly transverse in orientation as the leaf acquires its more mature linear shape. Subsequent leaves show a gradual restriction of division activity toward the blade base. A final peak in division occurs relatively late in development (L6/P7) at the base of the sheath after the blade stops dividing and differentiation is complete (Fig. 19F). The next leaf in the sequence (L5/P8) had stopped dividing entirely (data not shown).

The division orientation pattern corresponds with the change in leaf shape from ovate to linear, as depicted in Fig. 20B. The young leaves are spade-shaped with the widest portion at the base of the blade above the forming ligule. Epidermal anticlinal cell divisions in this region are longitudinal and transverse. As the leaf acquires more linear dimension, cell divisions are increasingly transverse in orientation.

A development index, used to represent the total proportion of leaf occupied by blade, emphasizes two times when the growth pattern changes (as indicated by the arrow and arrowhead; Fig. 20A). The first is when the preligule division band is first visible, also coinciding with the first visible differentiation of the blade and sheath cells (L10/P3; Fig. 20A, arrowhead). The second is when blade growth slows relative to sheath growth (between L8/P5 and L7/P6; Fig. 20A, arrow). These two timepoints divide development into three stages: (1) the preligular stage, (2) the stage of formation of the ligular region and rapid blade growth, and (3) the final stage of differentiation and rapid sheath growth.

The sheath grows little in length during the first two stages, although it continues to divide. Rapid increase in sheath length occurs in the last stage when blade and ligule growth have slowed (Fig. 20). Division is restricted to the base of the sheath near the end of development and final increase in leaf length is due to rapid extension and differentiation of the sheath cells. Cell differentiation parallels the changing course of division pattern. Initially, the blade is undifferentiated from the sheath at the preligular stage but as soon as the preligule band forms, cells at the tip of the adult blade differentiate hairs.

The distinction between growth of lgl/lgl and wild-type can be seen in the division pattern of sequential leaves (Fig. 21). Growth was slower in lgl/lgl in a W23 background as compared to our wild-type W23 line; that is, the first few plastochrons were characteristically smaller in the mutant. Consequently, a direct correspondence between Figs 19 and 21 is not possible. Nevertheless, peaks of division in the presumed ligule region of lgl/lgl are sometimes apparent. When present, these divisions are entirely transverse in orientation compared with the more frequent longitudinal divisions in the normal preligule division band. The change of division orientation is even more evident in older plants when a sporadic preligule band appears as clusters of cells derived from transverse-only and transverse plus longitudinal divisions (see previous results; Fig. 17C). Between leaves L6/P5 and L5/P6, divisions are restricted to the basal portion of the leaf. Divisions are also entirely transverse by this stage. Further increase in size must be due to cell extension, because at this stage 80% of the blade is not dividing. Division is restricted to the sheath base by leaf L4/P7 in the mutant. At this time, as in the wild-type, the sheath
Figs. 15–18. Pattern of ligule vestige formation in sequential leaves of $lgl/lgl$: an old plant.

Fig. 15. Leaf 6/Plastochron 8: mature adult leaf. Ligule vestiges are visible in several locations angling toward the mid-rib (B) or nestled between veins at the blade–sheath border (C).

Fig. 16. Leaf 7/Plastochron 7: immature adult leaf. A ligule ridge has formed but is localized rather than continuous and is unevenly distributed around the leaf (C; compare with Fig. 2D).

Fig. 17. Leaf 8/Plastochron 6: immature adult leaf. A normal preligule division band is present only sporadically around the leaf surface (arrow, C). Adjacent to a normal band is a region of transverse-only divisions (arrowhead, C).

Fig. 18. Leaf 9/Plastochron 5: immature adult leaf. There is no sign of a preligule division band despite differentiation of blade from sheath. Scale bar, 90 μm. All micrographs are at the same magnification except 15C (1 cm=275 μm), and 15D (1 cm=245 μm).

increases most rapidly in length. The $liguleless-1$ plant also shows the same overall pattern of leaf growth as wild-type (Fig. 22). The development index is similar regardless of whether ligule is absent entirely or whether ligule vestiges form (Fig. 22A). These results confirm that the mutation is specific to the ligule region and has little effect on the overall growth pattern of the leaf. The basipetal direction of maturation is also
Fig. 19. Division index of sequential leaves of a wild-type W23 plant. A recent cross-wall index is compared in each leaf. The number above each point refers to the percentage of transverse anticlinal divisions relative to total transverse and longitudinal anticlinal divisions. Vertical line represents the preligular or the ligular region (A) Leaf 11/Plastochron 2; (B) Leaf 10/Plastochron 3; (C) Leaf 9/Plastochron 4; (D) Leaf 8/Plastochron 5; (E) Leaf 7/Plastochron 6; (F) Leaf 6/Plastochron 7.

Fig. 20. Growth and differentiation in a wild-type plant. (A) Development index representing the ratio of blade length to total leaf length in sequential leaves of the same plant. The arrowhead indicates the time of preligule band formation. The arrow indicates when blade growth slows relative to sheath growth. Development is divided into three stages: (I) preligule stage before the division band forms; (II) blade growth stage when ligule grows out and blade differentiates; (III) sheath growth stage when blade stops growing. (B) Changing dimensions of blade (open squares) and sheath (closed triangles) are compared in sequential leaves of the same plant as in A. Distribution of waxes (shading on leaf surface) and differentiation of hairs (vertical lines) show that the transition from juvenile to adult is gradual over several plastochrons.

Discussion

Several distinct stages of growth of the maize leaf are described in this study. These are (1) the preligule stage, when the primordium is undifferentiated and dividing throughout its length, (2) the stage of ligule outgrowth and rapid blade growth, and (3) a stage of rapid sheath growth. Once the sheath completes extension the leaf has attained its final stature and is fully mature. It is significant that plant development involves at least three related events, including cell division (increase in cell number), cell extension (increase in cell size), and cell differentiation (acquisition of cell function). Traditionally, these events have been considered together and investigators have determined that growth is restricted to the base of the monocot leaf (Sharman, 1942; Esau, 1965; Kemp, 1980; Poethig, 1984). By investigating each aspect of growth individually, the present study permits more accurate conclusions. First, division is restricted to the base of the leaf only at a relatively late stage in development, typically well after differentiation of the blade is complete. The maize leaf does not have a basal leaf ‘meristem,’ because during 90% of leaf development the sheath divides along its entire length. Most of the increase in sheath length occurs late in the last stage of development and is due to localized cell division and cell extension. Second, localized regions of increased division are evident along the leaf surface, most notably in the preligule band, immediately above the ligule, and at the base of the sheath late in development. Third, orientation of division direction correlates with leaf
shape. At sites of increasing leaf width, as in young ovate leaves, divisions are primarily longitudinal in orientation, but transverse divisions predominate as leaves acquire the mature strap-shape. Sheath divisions are almost exclusively transverse. Fourth, orientation of division appears to be significant to normal ligule formation. The preligule anticlinal division peak is marked by a local increase in longitudinal followed by rapid transverse divisions. These division sequences contribute to the formation of orderly packets of small cells, which subsequently produce both the ligule and the auricle. In patches where only transverse divisions occur, ligule and auricle do not form.

Development of maize leaves involves two superimposed aging phenomena, that of relative leaf age and that of plant maturity. The distinct juvenile and adult morphology of the maize leaf exemplifies the chronological aging phenomenon. The concept of leaf age applies to how long a leaf has been growing since it emerged from the shoot meristem. Plant maturity, on the other hand, refers to the stage of the entire plant relative to the transition from the vegetative to flowering stage. Because the morphology of juvenile versus adult leaves are quite different, stages in their maturity may be assessed. Therefore, a given leaf has several developmental descriptions in addition to its leaf number and plastochron number: it may be juvenile or adult and mature or immature.

Using this system, we have shown that the transition from the juvenile to adult stage is gradual and spans at least 2 to 3 plastochrons of development. This provides information about the nature of the developmental signal. The trigger to change from juvenile to adult must be effective over a long time rather than acting locally during the production of a single leaf primordium. One interpretation of how this occurs is that any leaves immediately around the apex at the time the signal emanates are cued to produce new adult leaf features. In this case, waxes are no longer produced, hairs are initiated and the adult morphology ensues on all leaf parts that have not yet developed. This suggests that juvenile and adult features are not predetermined by position on the leaf or at some earlier stage of development. Presumably any alteration that prolongs or contracts the transition signal would change the pattern of transition from juvenile to adult. Studies on
Teopod mutants support these inferences. Teopod mutations appear to prolong the juvenile phase of growth and Tp1 was shown to act non-autonomously (Poethig, 1988).

By establishing this baseline of leaf growth, the effect of mutations to specific stages may be analyzed. The results presented here indicate that the recessive lgl mutation acts specifically during preligule and ligule formation stages. These results concur with a previous study of the developmental consequences of clonal sectors of liguleless tissue in normal leaves (Becraft et al. 1990). Becraft and co-workers showed that the Lgl1+ wild-type product is necessary in the epidermis for normal ligule to form and that normal ligules will grow from Lgl1+ epidermis even if the underlying mesophyll tissue is lgl1-. Our results show that the liguleless-1 gene does not influence the overall growth pattern: the basal direction of division and differentiation is similar to wild-type as is the general timing of maturity. The features that vary in the mutant are the predictable presence of the preligule division band, the orientation of divisions, and the effect of the state of maturity on the expression of the phenotype. Growth rate may be altered during early stages but this has not yet been shown. The peak of cell division that precedes normal ligule formation is most commonly absent from liguleless leaves or else is modified so that only transient transverse divisions appear in the juvenile leaves. In adult liguleless-I leaves, ligule vestiges can be predicted by the presence of normal preligule division bands of both longitudinal and transverse type interspersed with either no sign of increased division or division bands of the transverse-only type. Ligule vestiges show a distinct morphology. They often lie between veins forming pockets of ligule, or they angle in and up toward the mid-region. Where vestiges of ligule skip over veins they angle in toward the midrib with clear delineation of sheath from blade cells along the trajectory (as in Fig. 15C). However, leaves that lack ligule vestiges show a more gradual transition from sheath to blade cells. This is particularly evident in the distribution of waxes, which do not disappear abruptly at the presumed blade-sheath boundary.

It appears that a particular sequence of divisions precedes ligule formation. How might the Lgl1+ gene product be acting in this regard? It is not specifying the boundary between blade and sheath because the boundary is still present in the mutant: the overall ratio of blade to sheath remains constant even in the absence of the separating ridge of ligule and auricle tissue between them (compare Figs 20B and 21B). This implies that the ligule itself is not required to differentiate blade from sheath, despite the fact that in the normal condition these events all occur together. Observations of the distribution of ligule vestiges in old plants with adult leaves suggest the opposite may be true: ligule outgrowth will only occur at borders where blade and sheath cells are in contact. If the border between blade and sheath is altered so also will be the pattern of ligule outgrowth (shown here, J. Fowler, personal communication and Freeling et al. 1988). The conclusion is that the lgl mutation prevents normal communication at the blade-sheath border rather than altering the border itself. Without dividing properly (either in the anticlinal dimension, where longitudinal divisions are lacking, or in the periclinal dimension) a normal ligule does not form.

Does the Lgl1+ gene product then control the orientation of division planes? For this to be true the gene product would have to alter the cell rules (Green, 1980) that may dictate when a given orientation results from a given cell dimension. Becraft and co-workers (1990) showed that an increase in cell size in the periclinal dimension precedes the periclinal division during normal development but that such size changes were absent from the liguleless plants. Any rules that dictate when and where a cell will divide based on its size or shape must be encoded by genetic programs acting before the events we are recording. It is likely that the fundamental rules themselves have not been altered in the liguleless plants, but that the block occurs earlier than the ligule division stage. At this time we cannot give a causal role to a change in cell dimension as a determinate for ligule outgrowth, but we conclude that the communication block occurs before the preligule division band is initiated.

The integrity of the blade-sheath border appears crucial to subsequent developmental events. Several examples of this in liguleless plants are the differences in vascular pattern through the border (Becraft et al. 1990), and the presence of transverse-only anticlinal divisions in the preligule band. It is possible that the transverse-only anticlinal divisions described here might inhibit ligule outgrowth by producing more continuous cell files at the border rather than producing packets of cells that are segregated from their neighbors in the blade and sheath. The subsequent elongation of these cell files could carry the cells into the new blade above and sheath below thereby circumventing ligule outgrowth. A local region of longitudinal divisions, as in the preligule band, could serve to segregate the blade from sheath cells clonally.

The question remains as to when and how the blade-sheath border is determined during development. The lgl mutant is significant to our understanding of this question because it shows that the blade-sheath border: (1) is determined early in development before the ligular region is formed, (2) is present even in the absence of normal ligule formation, and (3) can be misplaced and still produce ligule vestiges. We now know that there are a number of interacting components that contribute to normal ligule outgrowth, including changes in local division rate and in orientation of division. How does the Lgl1+ gene product influence these components? The evidence presented here points to the lgl mutant being blocked in the communication of information sometime between when the border is determined and when the border shifts its division sequence.

The nature of the communication block in lgl homozygotes is of paramount importance to understanding the causal relationships in maize leaf develop-
The Lgl+ gene product may prevent the acquisition or interpretation of information required for the proper division sequence, thus preventing normal ligule outgrowth. Alternatively, the Lgl+ gene product may specify the information itself. This would likely occur early in development when the blade–sheath border is first forming. There is mounting evidence for a relationship between the pattern of vascular development and the subsequent differentiation of the border (data not shown). A number of other leaf developmental mutants that show alteration in ligule position or morphology also show concomitant changes in gross morphology of the midvein region. The midvein may be an organizing influence on general leaf morphology, starting with placement of lateral and intermediate veins and concluding with position of ligular tissue. The testing of these ideas awaits more information about other maize leaf mutants, a biochemical description of the Lgl+ protein, and direct experimentation with normal plant tissue to clarify when and how the blade–sheath border is determined.

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