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Leafbladeless1 is required for dorsoventrality of lateral organs in maize

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SUMMARY

The maize *leafbladeless1* (*lbl1*) mutant displays a variety of leaf and plant phenotypes. The most extreme manifestation in the leaf is the formation of radially symmetric, abaxialized leaves due to a complete loss of adaxial cell types. Less severe phenotypes, resulting from a partial loss of adaxial cell identity, include the formation of ectopic laminae at the boundary between abaxialized, mutant sectors on the adaxial leaf surface and the bifurcation of leaves. Ectopic laminae and bifurcations arise early in leaf development and result in an altered patterning of the leaf along the proximodistal axis, or in complete duplication of the developing organ. Leaf-like lateral organs of the inflorescences and flowers show similar phenotypes. These observations suggest that *Lbl1* is required for the specification of adaxial cell identity within leaves and leaf-like lateral organs. *Lbl1* is also required for the lateral propagation of leaf founder cell recruitment, and plays a direct or indirect role in the downregulation of the homeobox gene, *knotted1*, during leaf development. Our results suggest that adaxial/abaxial asymmetry of lateral organs is specified in the shoot apical meristem, and that formation of this axis is essential for marginal, lateral growth and for the specification of points of proximodistal growth. Parallels between early patterning events during lateral organ development in plants and animals are discussed.

Key words: Maize, *leafbladeless1*, Leaf initiation, Leaf development, Dorsoventrality, Meristem, Homeobox gene

INTRODUCTION

In higher plants, organogenesis continues beyond the period of embryogenesis, and lateral organs of the shoot develop progressively from the shoot apical meristem. As first steps in lateral organ formation, founder cells are recruited from the meristem and new developmental axes are established relative to the main body axis. The fate of the organ becomes determined gradually during primordium initiation and growth. Later in organogenesis, the appropriate cell types differentiate corresponding to the basic pattern and fate of the lateral organ.

Leaves are considered to be the ground state lateral organ, because specific homeotic mutations convert lateral organs, such as bracts, sepals and petals, into leaves (Coen and Meyerowitz, 1991; see Hagemann, 1984). Maize leaves are initiated through coordinate changes in cell division at a single point on the flank of the meristem (Sharman, 1942). Successive leaves are initiated on opposite sides of the meristem, resulting in a distichous (180°) phyllotaxy. The interval between the initiation of successive leaves is termed a plastochron. Consequently, the plastochron stage of a leaf describes its position relative to the meristem. For instance, a plastochron 3 (P3) primordium is the third leaf from the apex. The incipient leaf is referred to as P0. As the primordium grows, the altered cell division pattern is propagated laterally such that the base of the primordium forms a 'disc of insertion' that encircles the apex (Sharman, 1942). Clonal analysis has shown that approximately 200 founder cells in the shoot apex contribute to the development of the leaf and associated subtending node, internode and axillary bud (Poethig, 1984; Poethig and Szymkowiak, 1995). Together these four structures form the phytomer, or the repeating structural unit of the maize plant (Galinat, 1959).

The lack of expression of the homeodomain protein KNOTTED1 (KN1) marks the position of founder cells in the meristem (Jackson et al., 1994; Smith et al., 1992). KN1 is expressed throughout the meristem but is downregulated in leaves and leaf primordia. In addition, the expression of the *kn1*-related genes *rough sheath1* (*rs1*) and *knox3* at the base of the ring of founder cells suggests that these genes, together with *kn1*, may have a role in specifying and/or patterning of primordium initials (Jackson et al., 1994; Schneeberger et al., 1995). Consistent with this hypothesis, the *narrow sheath* (*ns*) mutant, which fails to establish leaf marginal domains, also fails to downregulate *kn1* expression in the corresponding subset of leaf founder cells (Scanlon et al., 1996).

Cell divisions occur uniformly throughout the P1-3 primordium, resulting in the development of a hooded primordium which encloses the apical meristem (Poethig, 1984; Sylvester et al., 1990). Subsequently, cell divisions become progressively more restricted to the base of the growing leaf, and cell differentiation proceeds basipetally (Poethig, 1984; Sharman, 1942; Sylvester et al., 1990). Little is known about the mechanisms by which the proximodistal,

lateral, and adaxial/abaxial axes are specified and interpreted in the developing leaf. The maize leaf comprises multiple domains along the lateral and proximodistal axes. Maize leaves are bilaterally symmetric and consist of a central midrib, middle blade regions and lateral marginal domains (Freeling, 1992). Observations made in the *ns* mutant suggest that not only lateral growth, but also the determination of these lateral domains may be established during founder cell recruitment (Scanlon et al., 1996).

Proximodistal growth begins first from the leaf initiation site, which later in development coincides with the position of the midvein (Sharman, 1942). From base to tip, the maize leaf comprises the sheath, ligular-auricular and blade regions. A model for the specification of domains along the proximodistal axis has been proposed based on the phenotypes of dominant mutations in several homeobox genes, including *Kn1* (see Hake, 1992), *Rs1* (Becraft and Freeling, 1994; Schneeberger et al., 1995), *Liguleless3* (Fowler et al., 1996; Muehlbauer et al., 1997) and *Liguleless4* (Fowler and Freeling, 1996). These mutations cause cells within the distal blade to acquire a more proximal sheath or auricle fate. According to the model, leaf founder cells progress through a series of competency states for differentiation (sheath-auricle-blade). A developmental signal spreads across the primordium and induces the fate of a cell to be determined based on its competency (Freeling, 1992; Muehlbauer et al., 1997). SEM analysis revealed the presence of ligular features in P3-4 primordia (Sylvester et al., 1990), suggesting that the interpretation of the proximodistal axis, as well as the specification of this axis, occurs early in leaf development.

The maize leaf is bifacial. The adaxial, or upper, leaf epidermis expresses several characters, including the ligule, macrohairs, and bulliform cells, which are absent in the abaxial, or lower surface. Adaxial/abaxial polarity is also visible in the internal leaf tissues. For instance, in vascular bundles, xylem is adaxially positioned relative to phloem, and in the midrib region, clear cells are adaxially positioned relative to the midvein. The presence of the ligule in P3 primordia indicates that adaxial/abaxial axis formation and patterning occurs during founder cell recruitment (P0) or during P1-2 primordium growth (Sylvester et al., 1990). Two dominant mutations, *Rolled1* (*Rld1*) and *Curled entangled1* (*Ce1*), affect adaxial/abaxial patterning in maize, resulting in the expression of adaxial characters on the abaxial leaf surface (Pawar and Mouli, 1973; Freeling, 1992). To date, no recessive mutations affecting adaxial/abaxial axis specification or patterning have been identified in maize. Insight into the possible phenotypes of such mutations comes from the morphology of unifacial leaves found in a number of divergent monocot species. Unifacial leaves frequently consist of a unifacial, radially symmetric tip with a bifacial and bilaterally symmetric base (Bharathan 1996; Kaplan, 1973).

Several recessive mutations affecting the dorsoventral morphology of leaves or leaflets have been described in dicot species. The *afilia* mutation in pea produces lateral tendrils in place of leaflets (Meicenheimer et al., 1983). In tobacco, *lam-1* leaves fail to develop laminae due to a cell division defect in the internal, L3 layer of newly initiated primordia (McHale, 1993). The severely reduced mutant leaves do maintain adaxial/abaxial asymmetry, however. In contrast, the *phantastica* (*phan*) mutant in *Antirrhinum* has a variable affect on leaf morphology, with radially symmetric, abaxialized leaves as the most extreme manifestation (Waites and Hudson, 1995). Less severely affected *phan* leaves frequently develop ectopic laminae on the adaxial leaf surface. Such outgrowths surround patches of abaxial epidermis. Based on these observations, Waites and Hudson (1995) concluded that *Phan* is required to establish adaxial cell identity, and that a defect in adaxial/abaxial axis formation results in a lateral growth defect.

Here we describe a recessive mutant of maize, *leafbladeless1* (*lbl1*), that exhibits defects in adaxial/abaxial patterning of lateral organs. In the absence of LBL1 activity, cells maintain an abaxial identity, which can result in the development of unifacial, thread-like leaves, in ectopic laminar outgrowths on the adaxial leaf surface, or in bifurcation of the leaf. Our results suggest that *Lbl1* is required for several aspects of leaf development, for the recruitment of founder cells in the meristem and for the determination of adaxial cell types. We propose that defects in the establishment of the adaxial/abaxial axis affect lateral growth and the specification of points of proximodistal growth. Parallels, therefore, exist between lateral organ development in plants and animals.

MATERIALS AND METHODS

Maize stocks and genetic analysis

The *lbl1* mutation was identified by D. Miles at the University of Missouri, Columbia, MO, USA (Miles, 1989). The *ragged seedling1* (*rgd1*) allele (Kramer, 1957) was obtained from Robert Martienssen at Cold Spring Harbor Laboratories, NY, USA. Inheritance of *lbl1* was analyzed by outcrossing wild-type and heterozygous siblings to several different inbred lines, self-pollinating the F_1 progeny, and scoring the frequency of *lbl1* mutant seedlings in the F_2 progeny. In outcrosses to inbred lines B73 and W22, the expected number of F_1 families (1/3) segregated *lbl1* seedlings, but the frequency of *lbl1* mutants per family varied from 7 to 22 percent. Similar results were obtained when backcross2 (BC2) or BC3 progeny into either B73 or W22 backgrounds were self-pollinated and screened for *lbl1* mutant segregation. The *lbl1* mutation, thus, segregates as a single recessive Mendelian trait, which exhibits weak penetrance in the B73 and W22 backgrounds.

Self-pollinated ears derived from BC2-B73 plants segregated for the endosperm marker *white endosperm* (*y1*), which is dosage-sensitive. When progeny from such ears were planted according to their *y1* dosage, linkage was observed between *lbl1* and *y1*. Due to the reduced penetrance of *lbl1* in B73, the map distance between *lbl1* and *y1* was calculated based only on the number of *lbl1* mutants in each of the four classes. The *lbl1-y1* map distance was found to be approximately 10 cM. Subsequently, the genetic map distance between *lbl1* and *y1* was confirmed by self-pollinating heterozygous *y1/+* plants derived from BC3-B73, and scoring the segregation of *lbl1* mutant seedlings among the progeny. Out of 51 self-pollinated ears that segregated *y1/+*, 2 did not segregate *lbl1* mutant seedlings. Because the *lbl1* mutation results in male sterility, only one class of recombinant chromosomes could be identified in this analysis. The map distance between *y1* and *lbl1* in this experiment is therefore approximately 8 cM.

Allelism between *lbl1* and *rgd1*, which exhibit similar phenotypes, was tested by self-pollinating plants heterozygous for *rgd1/+* and outcrossing the same plants to weakly phenotypic, female fertile *lbl1* mutant plants. Progeny from such crosses segregated a phenotype that resembled the weak *lbl1* mutant phenotype.

DNA isolation and Southern analysis

Genomic DNA from leaf samples was isolated as described by Das et al. (1990). Southern blots were prepared and hybridized according to Timmermans et al., (1996). Probes were isolated with the Gene-Clean kit (Bio 101) and labeled by random priming. A cDNA clone of the *y1* gene was provided by B. Buckner, Northeast Missouri State Univ., Kirksville, MO, USA (Buckner et al., 1996).

In order to place the *lbl1* locus proximal or distal of *y1* on chromosome 6, recombination frequencies between the *lbl1*, *y1* and *umc85* loci were determined by restriction fragment length polymorphism (RFLP) linkage analysis. A total of 65 *lbl1* mutant seedlings obtained by self-pollinating BC2-B73 or BC2-W22 plants were analyzed. The following map distances were calculated: *y1-lbl1*, 2.3 cM; *lbl1-umc85*, 1.5 cM; *y1-umc85*, 3.8 cM. These observations placed the *lbl1* locus proximal to *y1* on chromosome 6, in close proximity to *rgd1*.

Histology

Tissues for histological analysis were fixed and dehydrated as described by Jackson (1991). Following infiltration with propylene oxide, the tissues were embedded in Spurr's resin (Polysciences, Warrington, PA). 1 μm sections were cut using glass knives and stained for 1 minute in an aqueous 0.05% toluidine blue solution. Sections were mounted in a 3:1 mixture of Permount and xylene and photographed under bright-field conditions on a Zeiss Axiophot microscope.

Immunohistology

Shoot apices of 2-week old *lbl1* and wild-type sibling seedlings were fixed and embedded as described previously (Jackson, 1991). Immunohistochemistry using a KN1-specific polyclonal antibody (kindly provided by S. Hake, Plant Gene Expression Center, Albany, CA) was performed as described by Smith et al. (1992). Alkaline phosphatase conjugated goat-anti-rabbit-IgG (Boehringer Mannheim) diluted 1:600 in PBS/BSA was used as a secondary antibody, and immunopositive nuclei were visualized by incubation with the substrate 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt/nitroblue tetrazolium chloride (Boehringer Mannheim) overnight at room temperature. Slides were rinsed in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8) and counterstained with basic fuchsin according to Smith (1994). In order to compare approximately equivalent P0 stages, the length of the P1 primordium was estimated by counting the number of 8 μm sections from the tip of the P1 primordium to the site of the P0 incipient leaf.

Scanning electron microscopy

Apices from wild-type and *lbl1* mutant siblings were dissected and fixed overnight at 4°C in FAA (50% ethanol, 5% glacial acetic acid, 5% formalin). Tissues were dehydrated through an ethanol series, 35-100%, and critical point dried. Specimens were coated with a mixture of gold and palladium in a SPI sputter coater, and analyzed on an ISI-SS40 scanning electron microscope using an accelerating voltage of 10 kV.

Epidermal impressions

The epidermis of wild-type and *lbl1* mutant leaves was analyzed by making epidermal impressions in cyanoacrylate glue as suggested by L. Smith, Univ. of North Carolina, Chapel Hill, NC, USA. Briefly, leaf samples were placed on a drop of glue (Quick Tite super glue) on glass slides. Leaf tissues were removed after the glue had dried, and slides were examined using DIC optics on a Zeiss Axiophot microscope.

RESULTS

Morphology of *lbl1* mutant plants

The *lbl1* mutation segregates as a single recessive trait, but the penetrance and expressivity of the mutation are variable and dependent on genetic background and temperature (Fig. 1). The *lbl1* mutant phenotype is weak after outcrossing into the inbred lines B73 and W22 for two or more generations, whereas a more severe phenotype is visible in the *Mutator* transposon stock in which *lbl1* arose. Mutant plants grown at 30°C also display a stronger phenotype than plants grown at 26°C (Miles, 1989).

Weakly phenotypic *lbl1* mutant plants show a variable loss of leaf laminae, ranging from normal to narrow leaves or leaves with half a blade region (Fig. 1A-C). Severity of the leaf phenotype varies between plants as well as among leaves of a single plant. The flagleaf, defined as the uppermost leaf immediately below the tassel, is generally the most strongly affected. Mutant leaves often bifurcate along the midrib or develop ectopic outgrowths on the adaxial leaf surface (Fig. 1C,F; see below). The stature of weak *lbl1* mutant plants is similar to nonmutant siblings, in that both internode elongation and phyllotaxis appear normal.

A severe *lbl1* mutant phenotype is visible in the coleoptile, which is narrow or bifurcated. Within the plant, all components of the phytomer are affected (Fig. 1D). Leaves are extremely narrow or thread-like, and the internodes are shortened and curved. Mutant leaves are shorter, and are typically wider at the base than at the tip. Wild type leaves develop a ligule on the adaxial leaf surface at the boundary between sheath and blade tissue (Fig. 1E). Thread-like *lbl1* leaves develop a narrow ligular fringe, although occasionally the ligule is missing (Fig. 1G).

The degree of internode shortening and curving in *lbl1* mutant plants is correlated with the width of the associated leaf. Leaves are derived from the upper region of the disc of insertion, whereas the base of the disc of insertion contributes to the outer cell layers of the subtending internode (Sharman, 1942). The internal pith tissue of the culm is derived from the rib meristem. In normal phytomers, the disc of insertion surrounds the shoot apex, causing the outer cell layers of the stem to encircle the culm pith. In *lbl1*, the width of the outer internode layers is reduced similar to the leaf, exposing the culm pith, and reducing internode development and girth in regions opposite the narrow leaf insertion (data not shown). In the most severe mutant plants, the internodes fail to elongate and leaves have difficulty emerging (Fig. 1D). Such plants show a reduced apical dominance, and axillary meristems develop further than in wild-type siblings. The time to flowering is delayed in such plants, and the number of leaves produced is higher. The phyllotaxis remains distichous even in the most severe *lbl1* mutants (Fig. 1D).

The leaf-like organs of the flowers and the female inflorescence are abnormal in *lbl1* mutants, but the arrangement of spikelets on the male and female inflorescence is unaffected (Fig. 2). Husk leaves on severe *lbl1* mutant plants develop as radially symmetric, thread-like organs, and the immature ear is exposed and arrested in development (Fig. 2A,B). Less severe *lbl1* plants are female fertile. In contrast, all *lbl1* mutants are male sterile. In severe *lbl1* mutant plants, tassel glumes, lemmas and paleas develop as radially symmetric organs, and anther development is arrested at an early stage (Fig. 2C,D). In less severe *lbl1* mutant plants, development of glumes, lemmas and paleas appears mostly normal. However, anther development remains abnormal,

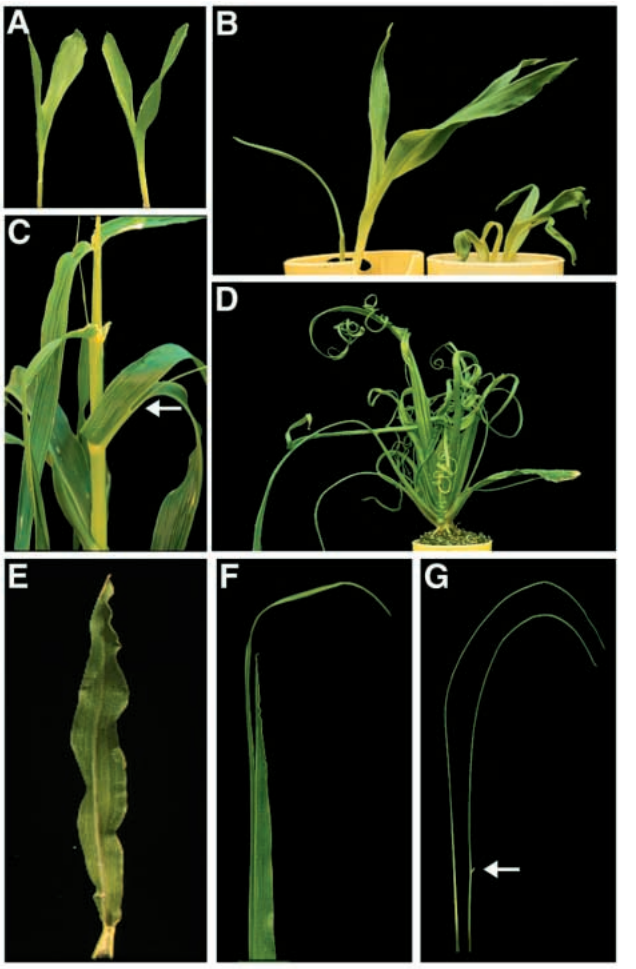

Fig. 1. Morphology of *lbl1* mutant plants. (A) Wild-type maize seedling. (B) *lbl1* mutant seedlings. *lbl1* mutant leaves show variable laminar development. (C) Weakly phenotypic *lbl1* mutant plant. Mutant leaves appear nearly normal in width, but they frequently bifurcate along the midrib (arrow). Internode elongation and phyllotaxis are unaffected. (D) Strongly phenotypic *lbl1* mutant plant. Mutant leaves are narrow, or thread-like. Internodes fail to elongate, but the phyllotaxis remains distichous. (E) Wild-type leaf consisting of a distal blade and proximal sheath region, which are separated by the ligule-auricle region. The ligule develops on the adaxial leaf surface. (F) Weakly phenotypic *lbl1* mutant leaf. Laminar development varies on either side of the midrib, and the leaf is bifurcated at the tip. (G) Severe phenotypic *lbl1* mutant leaves. The most severe *lbl1* mutant leaves are radially symmetric. Such thread-like leaves usually develop a reduced ligular fringe (arrow), however, occasionally the ligule fails to develop.

pollen production is strongly reduced and the pollen are non-viable (data not shown). In addition, spikelets fail to open at anthesis, suggesting that lodicule development in *lbl1* mutants is also affected.

The *lbl1* mutation affects adaxial/abaxial patterning and lateral growth of the leaf

Maize leaves develop adaxial/abaxial asymmetry both in the arrangement of laminae around the midrib, and in the patterning of the laminae and midrib (Fig. 3A,B). Laminae are located at the base of the midrib, near the midvein. In the midrib region, clear cells differentiate adaxial and sclerenchyma abaxial relative to the midvein. Within the midvein and in the vascular bundles of the laminae, xylem elements differentiate on the adaxial side of phloem tissue. Moreover, the presence of the ligule, macrohairs and bulliform cells distinguishes the adaxial blade epidermis from the abaxial epidermis.

The most severe *lbl1* mutant leaves fail to develop flattened laminae and have a radially symmetric, thread-like morphology. They typically develop a narrow ligular fringe (Fig. 1G), suggesting that adaxial cell files are present at the sheath-blade boundary. Consistent with the reduced ligule width, two rows of marginal hairs develop in close proximity to each other, delimiting a narrow region with adaxial cell files (Fig. 3E). The epidermis of distal blade regions is devoid of marginal hairs, macrohairs and bulliform cells, indicating that such thread-like *lbl1* leaves are unifacial and have an abaxial epidermis (Fig. 3F). Transverse sections through the base of radial *lbl1* leaf blades show a circular arrangement of vascular bundles around clear cells (Fig. 3C). The adaxial/abaxial patterning within the vascular bundles appears unaffected here, and the veins are arranged in centrifugal fashion such that their xylem poles are oriented towards the center of the leaf. Transverse sections through more distal blade regions, however, show a complete loss of asymmetry. The *lbl1* leaves consist of a central, irregular vascular cylinder surrounded by concentric rings of bundle sheath, mesophyll and abaxial epidermis (Fig. 3D). Even though the vascular bundle still contains xylem and phloem elements, these tissues are no longer organized in distinct poles. Despite this, the vascular tissue is surrounded by bundle sheath and mesophyll cells in the Kranz configuration typical of C4 tissues. Based on these observations, the thread-like *lbl1* mutant leaves could result from a defect in lateral growth with a subsequent loss in adaxial/abaxial patterning, or vice versa.

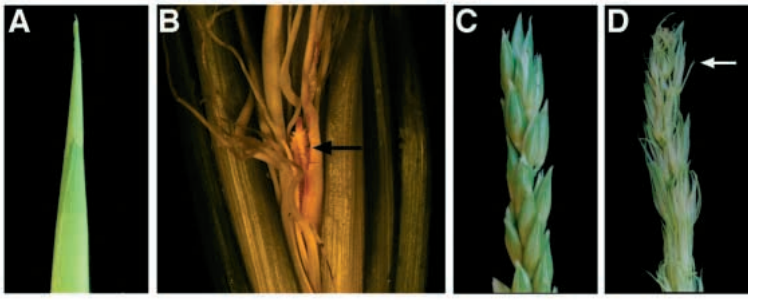

Fig. 2. The *lbl1* mutation affects floral organ development. (A) Wild-type female inflorescence. The ear is fully enclosed by husk leaves. (B) Female inflorescence on a severe phenotypic *lbl1* mutant plant. The width of husk leaves is strongly reduced and the female inflorescence is exposed (arrow). (C) Wild-type male inflorescence. Pairs of florets are enclosed in the glumes of a spikelet, which are arranged in pairs along the tassel branches. (D) Tassel branch of a strongly phenotypic *lbl1* mutant plant. Spikelet glumes (arrow) and male floral organs are reduced in size, and the plant is male sterile. The arrangement of spikelets on the inflorescence is unaffected.

Lbl1 is required for adaxial cell identity

The development of ectopic laminae on the adaxial leaf surface of weakly phenotypic *lbl1* mutant leaves is consistent with the interpretation that *Lbl1* is required for adaxial/abaxial patterning (Fig. 4A). Transverse sections revealed that ectopic outgrowths develop in pairs that flank a tissue sector with mainly abaxial characters (Fig. 4B). Frequently, the ectopic outgrowths flank the midvein. In such cases, the midvein still develops a xylem and phloem pole and abaxial sclerenchyma, but adaxial clear cells are absent or reduced in number, and the main laminae are initiated from a more adaxial position. The ectopic outgrowths resemble laminar tissue and develop a suite of marginal characters, including a tapered morphology, sclerenchyma cells, and sawtooth marginal hairs (Fig. 4B). The epidermis flanked by the laminar outgrowths lacks bulliform cells and macrohairs and thus appears abaxial (Fig. 4D,F). In contrast, the outer epidermis of the ectopic laminae exhibits normal adaxial characters (Fig. 4C,E). Consistently, the xylem elements of the vascular bundles in the ectopic outgrowths are oriented towards the outer epidermis. These observations suggest that the ectopic laminae occur at the boundary of abaxialized sectors on the adaxial leaf surface. This aspect of the *lbl1* mutant phenotype taken together with the lack of lateral growth and the abaxial nature of severe *lbl1* mutant leaves, is not consistent with a direct role for *Lbl1* in lateral growth. Both phenotypes are consistent, however, with *Lbl1* being required for establishing adaxial cell identity. In this case, lateral growth would result from the juxtaposing of adaxial and abaxial ‘domains’. In the absence of LBL1 activity, the primordium becomes radially symmetrical and the epidermis maintains an abaxial identity.

The ectopic laminae develop near the tip of mutant leaves and usually at or in close proximity to the midrib (Fig. 4A). Because the midvein develops early during leaf morphogenesis, the occurrence of lateral growth prior to the complete adaxial patterning of the midvein region suggests that the level of or requirement for an adaxial signal may vary in different regions of the primordium.

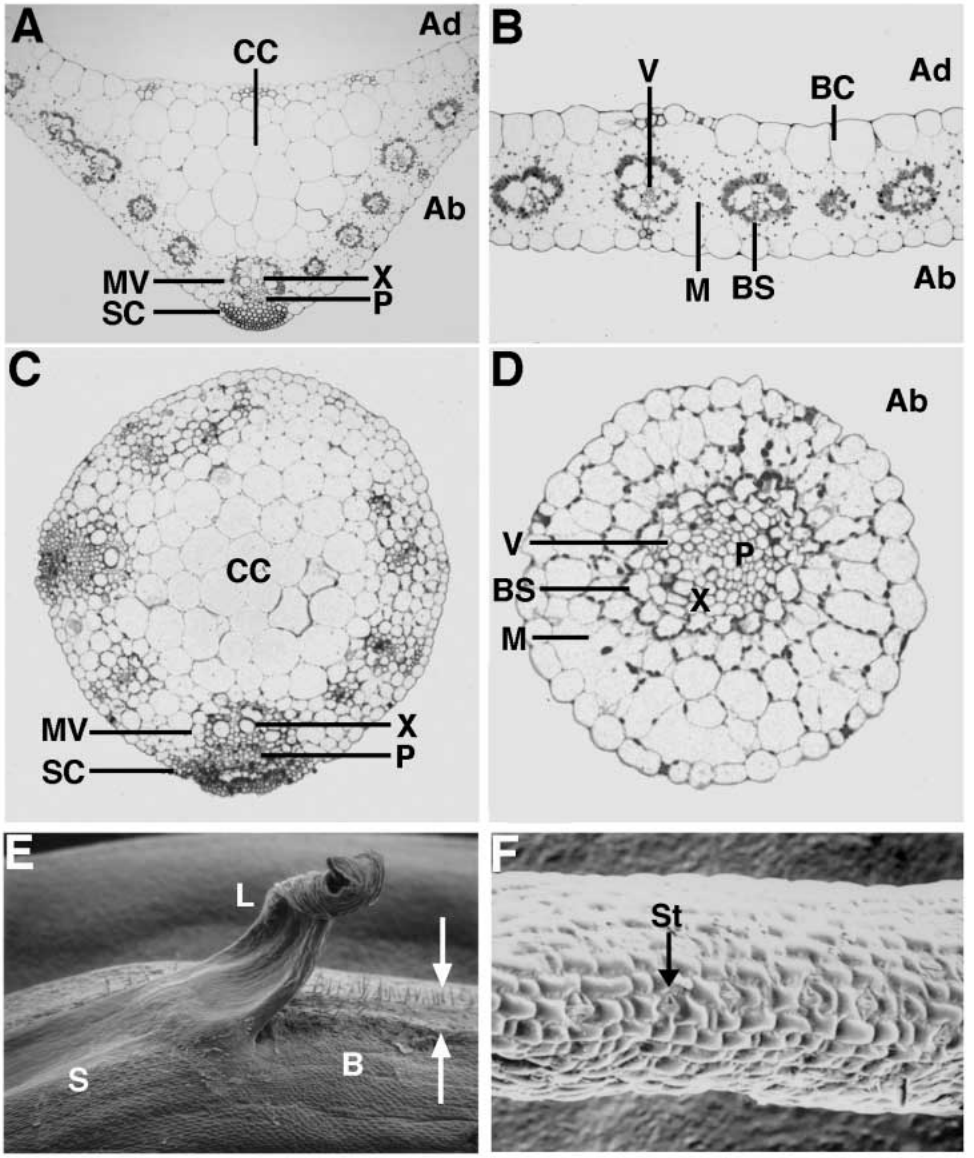

Fig. 3. Anatomy of severe *lbl1* mutant leaves. (A) Transverse section through the midrib region of a wild-type leaf. Laminae are adaxially positioned relative to the midvein. Clear cells and sclerenchyma differentiate adaxial and abaxial to the midvein, respectively. Within the vascular bundles, xylem differentiates adaxially to phloem. (B) Transverse section through the wild-type leaf blade. Veins are surrounded by bundle sheath and mesophyll cells. Bulliform cells differentiate in the adaxial epidermis only. (C) Transverse section through the proximal region of a radially symmetric *lbl1* leaf blade. Severe *lbl1* leaves develop no dorsoventrality. Instead, vascular bundles are radially arranged around the clear cells of the midvein region. The epidermis is abaxial, but adaxial/abaxial asymmetry is still apparent within the vascular bundles. (D) Transverse section through the distal region of a radially symmetric *lbl1* leaf blade. The *lbl1* mutant leaf consists of an irregular vascular cylinder surrounded by concentric rings of bundle sheath, mesophyll cells and abaxial epidermis. (E) SEM of the ligular region of a severe *lbl1* mutant leaf. The number of adaxial cell files is strongly reduced. As a result, two rows of marginal hairs develop in close proximity to each other and flank a reduced ligule (arrows). (F) SEM of the distal blade region of a radially symmetric *lbl1* leaf. The epidermis is completely abaxial and void of marginal hairs, macrohairs, and bulliform cells. Ad, adaxial; Ab, abaxial; CC, clear cells; MV, midvein; SC, sclerenchyma; P, phloem; X, xylem; V, vein; BC, bulliform cells; M, mesophyll; BS, bundle sheath; L, ligule; S, sheath; B, blade; St, stoma.

For instance, *Lbl1* may be predominantly expressed on the flanks of the growing primordium, or higher levels of LBL1 activity may be required for adaxial patterning of the midvein region. The length of the ectopic laminae can vary, possibly because the level of *Lbl1* gene function varies among leaves. Most frequently, ectopic laminae affect just the leaf blade, but they can extend well into the leaf sheath.

Loss-of-function mutations in *kn1* can also result in the formation of whole or partial ectopic leaves (Kerstetter et al., 1997). The polarity of these leaves is inverted, such that the adaxial surfaces of the ectopic and normal leaves face each other. Occasionally, the ectopic leaf is fused to the subtending main leaf and may thus resemble the ectopic laminar outgrowths observed in *lbl1*. However, ectopic leaves in *kn1* mutants are inserted into the node of the associated normal leaf and often do not extend the full length of the main leaf. They are therefore proposed to arise from the ectopic recruitment of founder cells. In contrast, the ectopic laminae in *lbl1* extend basipetally from the tip of mutant leaves, and are not likely to result from ectopic founder cell recruitment but rather from ectopic lateral growth of the primordium after initiation.

The *lbl1* mutation affects early steps in leaf development

The *lbl1* phenotype is most pronounced at the tip of the leaf, consistent with a role for *Lbl1* early in leaf development. Moreover, severe *lbl1* mutant plants exhibit a reduced width in the leaf as well as in the internode, suggesting that *lbl1* may affect founder cell recruitment. SEM of *lbl1* mutant apices confirmed that the morphology of young leaf primordia is affected in *lbl1* (Fig. 5). The base of a wild-type primordium encircles the apical meristem, and a P3 primordium fully encloses the shoot apex (Fig. 5A). In contrast, *lbl1* mutant primordia frequently fail to enclose the apical meristem even at later plastochron stages, and they exhibit a limited region of insertion, rarely surrounding the apex (Fig. 5B,C). The morphology of strongly phenotypic *lbl1* mutant primordia resembles that of some monocot species that develop a Vorläuferspitze (Bharathan 1996; Kaplan, 1973). Such primordia develop a radially symmetric tip but develop bilateral asymmetry towards the base (Fig. 5C).

Founder cells within the shoot apical meristem can be identified by the absence of *kn1* expression. Downregulation of *kn1* RNA in founder cells is evident early in P0, but KN1 protein is not completely downregulated till late in P0 (Jackson et al., 1994; Smith et al. 1992). Immunolocalization using a KN1-specific antibody on transverse sections of wild-type apices at the level of the incipient leaf show the accumulation of KN1 protein in the meristem proper (Fig. 5D). Cells within the new P0 primordium no longer accumulate KN1, with the exception of some cells in the pre-marginal region. At the level of the disc of insertion of the P1 primordium, a doughnut-shaped ring of cells no longer express *kn1* (data not shown). The region in which cells do not accumulate KN1 protein is far smaller in *lbl1* mutant apices, both at the level of the incipient leaf and at the level of the P1 disc of insertion (Fig. 5F). In both mutant and wild type, within the domain of cells not accumulating KN1, expression is uniformly off. Besides the altered *kn1* expression pattern, no other histological or morphological differences are apparent in *lbl1* mutant meristems. Therefore, the *lbl1* leaf morphology does not result from a defect in meristem organization, nor from a failure to uniformly downregulate KN1 in the incipient leaf. Rather, *Lbl1* seems to be required to propagate the recruitment of leaf founder cells from the meristem.

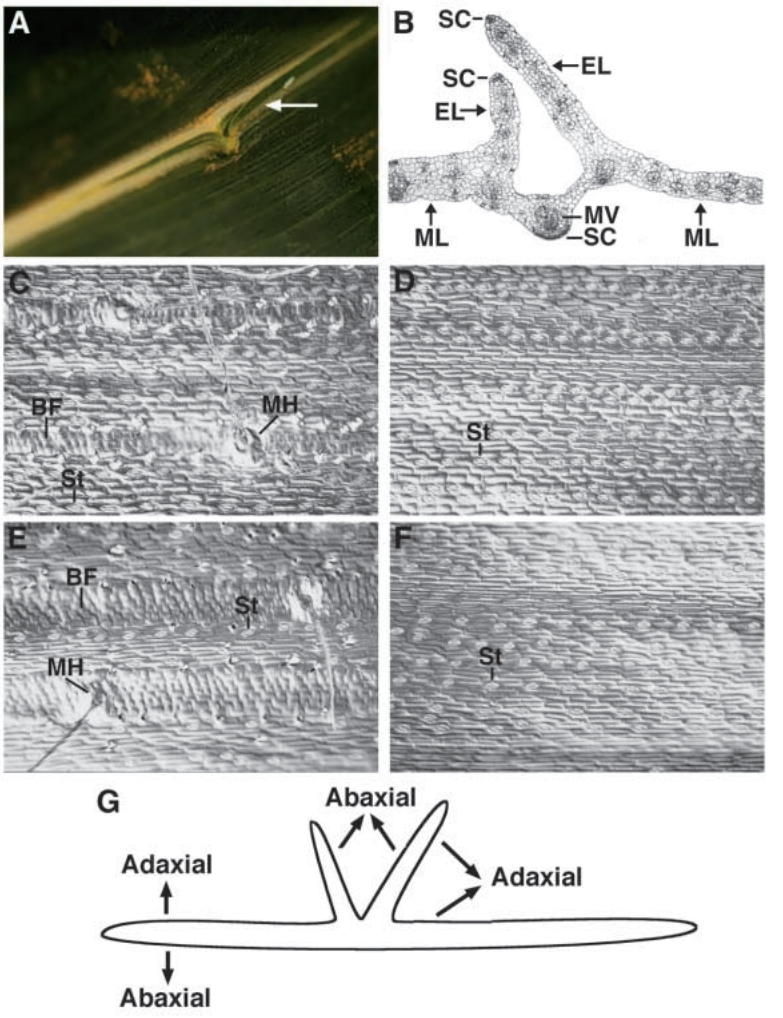

Fig. 4. Morphology of weakly phenotypic *lbl1* mutant leaves. (A) Ectopic outgrowths (arrow) develop on the adaxial leaf surface of weakly phenotypic *lbl1* leaves, near the midrib and basipetally from the leaf tip. (B) Transverse section through ectopic outgrowths. Pairs of ectopic laminae flank a tissue sector expressing fewer adaxial characters. Their morphology resembles normal leaf laminae, including features of wild-type margins. (C-F) Epidermal impressions. (C) The adaxial blade epidermis contains rows of bulliform cells and macrohairs. (D) The abaxial blade epidermis is void of these characters. (E) The outer epidermis of the ectopic laminae contains bulliform cells and macrohairs and is thus adaxial in nature. (F) The epidermis between the ectopic outgrowths resembles the abaxial blade epidermis. (G) Schematic diagram of the morphology of the ectopic laminae on weakly phenotypic *lbl1* leaves. EL, ectopic laminae; ML, main laminae; MV, midvein; SC, sclerenchyma; BF, bulliform cells; MH, macrohairs; St, stoma.

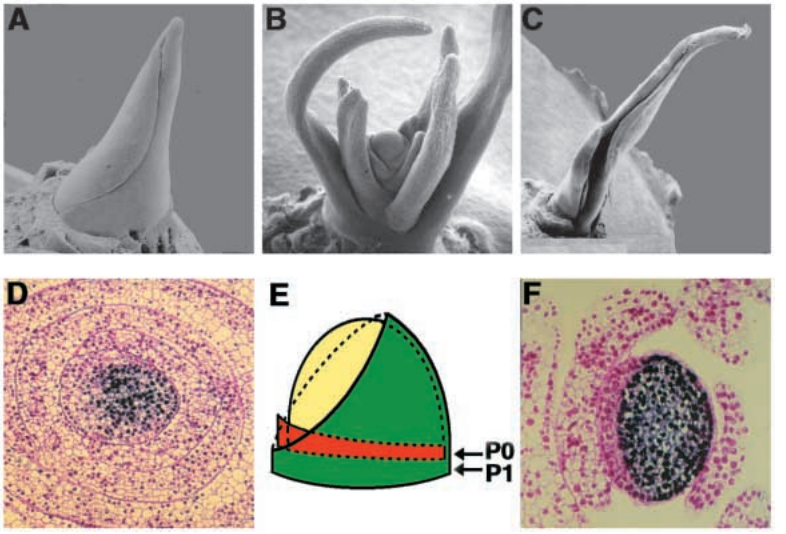

Fig. 5. Leaf morphogenesis in the *lbl1* mutant. (A) SEM of a wild-type shoot apex. The disc of insertion of wild-type primordia encircle the apex and the P4 primordium fully encloses the meristem. (B,C) SEM of a *lbl1* mutant apices. *lbl1* primordia frequently fail to enclose the meristem, and the region of insertion is reduced (B). The apparent unusual phyllotaxy is a consequence of leaf bifurcation subsequent to initiation. The tips of *lbl1* mutant primordia are usually more severely affected (C). (D) Immunohistochemical analysis of KN1 accumulation in a wild-type meristem. KN1 is expressed throughout the meristem proper (purple nuclei), but not in leaf primordia or in the incipient leaf (pink nuclei). (E) Schematic diagram of the arrangement of leaf primordia around a wild-type shoot apex. Arrows indicate the approximate position of transverse sections to analyze the P0 and P1 disc of insertion. (F) Immunohistochemical analysis of KN1 accumulation in a *lbl1* mutant meristem. The number of cells not accumulating KN1, i.e. the number of leaf founder cells, is strongly reduced.

Effects of *lbl1* on proximodistal axis specification

Weakly phenotypic *lbl1* mutant leaves frequently bifurcate (Fig. 6). Like ectopic laminae, bifurcations usually arise near the midrib and can affect just the leaf tip or sometimes the entire leaf. Occasionally, the bifurcations in *lbl1* leaves result in the formation of ectopic thread-like outgrowths indicating that a relatively small number of cells can be partitioned from the remainder of the leaf primordium (Fig. 6D). Leaves that develop ectopic laminae over a long portion of the leaf are frequently bifurcated at the tip (Fig. 6C). The formation of ectopic laminae or bifurcations affects the specification of the proximodistal axis. In the wild-type leaf, a single proximodistal axis is established and the ligule-auricle region forms a sharp demarcation across the leaf between the sheath and blade. In *lbl1* mutant leaves, development of bifurcations or ectopic laminae that extend into the leaf sheath result in the independent placement of ligule and auricle tissue on either side of the bifurcation/ectopic laminae (Fig. 6A). Some leaves exhibit multiple small patches of ligular tissue that are surrounded by ectopic laminae (Fig. 6B). The ectopic differentiation of margins, thus, isolates small groups of cells within the primordium that respond independently to positional cues. A single leaf was found that had developed a midrib in each half leaf (Fig. 6C).

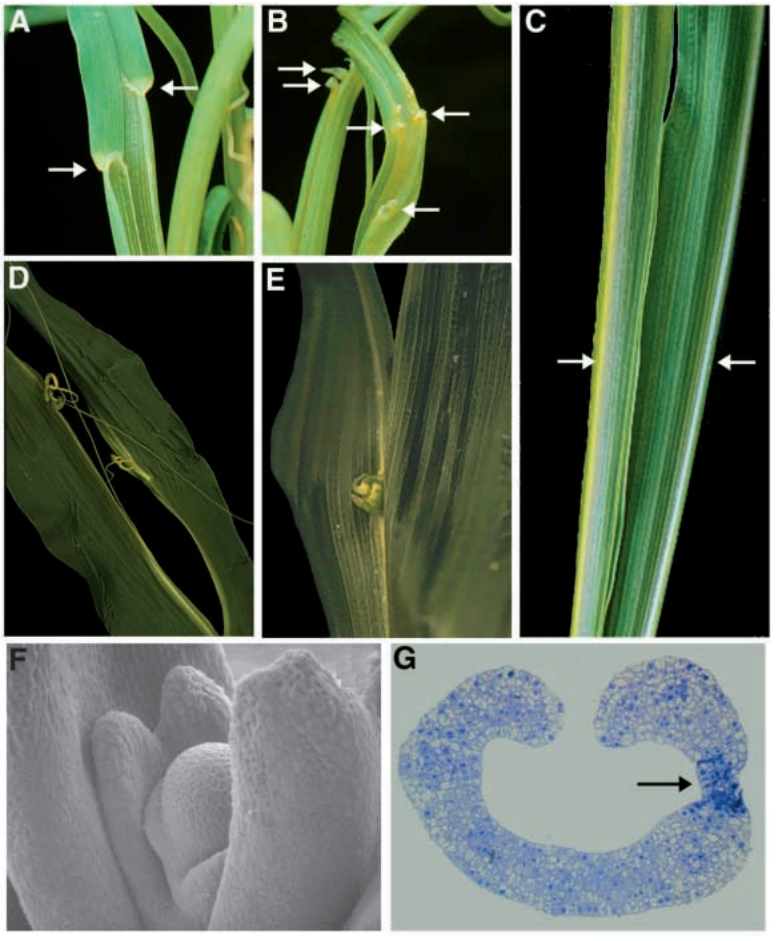

Fig. 6. *lbl1* affects proximodistal axis specification. (A,B) Altered ligule/auricle development on weakly phenotypic *lbl1* mutant leaves. Development of the ligule/auricle region on either side of ectopic laminae or bifurcations occurs at different positions along the proximodistal axis of the leaf (A). Some leaves develop multiple ectopic margins and multiple ligular patches (B). (C) Altered midrib development on weakly phenotypic *lbl1* mutant leaves. A single bifurcated leaf was found that developed two midribs. (D,E) Development of knot-like structures on weakly phenotypic *lbl1* mutant leaves. Knot-like structures occasionally develop on the margins of bifurcated leaves. Such knots are associated with additional radially symmetric outgrowths (D). Knots usually develop at the base of bifurcations and ectopic laminae (E). (F) SEM of a *lbl1* mutant apex. The P3 primordium has developed two distinct points of proximodistal growth, indicating that bifurcations develop early in leaf morphogenesis. (G) Transverse section through a *lbl1* primordium just below the point of bifurcation. The region below the point of bifurcation contains more densely staining cells (arrow).

Mature, adult *lbl1* leaves develop knot-like protrusions at the base of bifurcations and ectopic laminae (Fig. 6E). The knots form blade tissue on the adaxial side of the leaf, ligule in the plane of the leaf, and auricle or sheath-like tissue on the abaxial side of the leaf. The latter two characteristics are observed in the knots of dominant *Kn1* mutants (Hake, 1992). Weakly phenotypic *lbl1* mutant primordia exhibit bifurcations already in P3 (Fig. 6F). In transverse sections of bifurcated primordia, densely staining cells were observed at the base of the bifurcation (Fig. 6G). The nature of these cells is unclear, although the fact that their position coincides with the position of the knots later in leaf development suggests that these cells may be less determined and more meristematic.

The *raggedseedling1* (*rgd1*) mutation is an allele of *lbl1*

Self-pollinated families derived from outcrosses of heterozygous *lbl1/+* plants to inbred lines B73 and W22 revealed linkage between *lbl1* and the endosperm marker *white endosperm1* (*y1*), placing the *lbl1* locus on chromosome 6 approximately 9 cM from *y1*. Subsequent RFLP linkage analysis placed *lbl1* between *y1* and *umc85*, and in close proximity to *rgd1* (Materials and Methods). Allelism tests confirmed that *rgd1* is an allele of *lbl1*. Compared to the weak *lbl1* allele, the *rgd1* mutation causes a more uniform and severe phenotype, which frequently results in embryo lethality (data not shown). *rgd1* mutant leaves generally develop a radially symmetric, thread-like morphology without bifurcations. However, the first few leaves often display a weaker phenotype, including the formation of ectopic laminae. *rgd1* mutant plants are both male and female sterile.

DISCUSSION

The *lbl1* mutation affects plant and leaf morphology in a variety of ways. The most extreme manifestation in the leaf is the formation of radially symmetric, abaxialized leaves. Less severe phenotypes include the formation of ectopic laminae that express all marginal characters, at the boundary of abaxialized sectors on the adaxial leaf surface, and the bifurcation of leaves. Leaf-like lateral organs in the inflorescences and flowers display phenotypes similar to those observed in the leaf. Furthermore, *lbl1* mutant plants have defects in anther development and produce non-viable pollen. The tapetum layer is the adaxial tissue layer in the locule and is essential for pollen maturation. Because *lbl1* is a recessive mutation, these observations are consistent with a role for *Lbl1* in establishing adaxial cell identity in the leaf and other dorsoventral lateral organs. *Lbl1* is required for the propagation of founder cell recruitment, for lateral growth of the primordia, and for the determination of adaxial cell types.

Comparison between *lbl1* and the *Antirrhinum phan* mutant

The *phan* mutation in *Antirrhinum* exhibits similar defects in adaxial/abaxial patterning and results in the loss of adaxial cell identity in leaves, sepals and petals (Waites and Hudson, 1995). However, several important differences are apparent between the *lbl1* and *phan* mutant phenotypes. Firstly, *lbl1* affects all lateral organs that develop adaxial/abaxial asymmetry, whereas development of the sepals and tapetum layer are unaffected in *phan*. Secondly, thread-like *lbl1* leaves resemble lateral blade tissue, whereas pinform *phan* leaves resemble the midrib region of the wild-type leaf in that their central vascular bundle is surrounded by parenchyma and abaxial epidermis of the normal midrib. Thirdly, the *lbl1* mutant phenotype is typically more pronounced at the leaf tip: ectopic laminae initiate in distal regions while adaxial/abaxial asymmetry is often restored towards the leaf base. In contrast, the *phan* mutant phenotype is more severe in proximal regions of the leaf: ectopic laminae occur near the base of weakly phenotypic leaves and pinform *phan* leaves often develop dorsoventrality towards the leaf tip. Fourthly, unlike *Phan*, *Lbl1* is required for the propagation of founder cell recruitment in the meristem beyond the point of initiation. Lastly, *lbl1* mutant leaves frequently bifurcate at the tip, and establish multiple proximodistal axes. The *phan* mutation has no apparent effect on the specification of points of proximodistal growth.

Some of these phenotypic differences, such as the effect on founder cell recruitment, may reflect differences in lateral organ development between monocot and dicot species. The lateral growth of dicot leaves occurs largely subsequent to initiation and laminae develop on the flanks of the primordium. The lateral growth of monocots leaves is largely the consequence of the lateral recruitment of founder cells around the shoot apex in P0. Alternatively, it may be possible that such differences in monocot and dicot leaf development result from differences in the expression patterns of genes like *Lbl1* and *Phan*. Other phenotypic differences between *lbl1* and *phan*, such as the effect on proximodistal axis specification, pollen development, and leaf morphology suggest differences in the mode and/or site of action between *Lbl1* and *Phan*. This is consistent with our recent observation that the maize homolog of *Phan* is not *Lbl1*, but rather *Rough sheath2* (*Rs2*) (M. Timmermans, A. Hudson, P. Becraft, and T. Nelson, unpublished results). Like *phan*, *rs2* mutant plants produce viable pollen and the leaf phenotype is most pronounced towards the base, at the sheath-blade boundary.

The recently described *ARGONAUTE* (*AGO1*) gene from *Arabidopsis* represents another potential component in the dorsoventral patterning of leaves (Bohmert et al., 1998). *ago1* mutants produce filamentous and bifurcated lateral organs, but the presence of ectopic marginal outgrowths has not been reported.

Proposed roles of LBL1 in leaf development

The range of leaf phenotypes observed in *lbl1* mutant plants suggest that *Lbl1* establishes or maintains adaxial cell identity at critical times from leaf initiation through leaf cell differentiation (Fig. 7). Leaves are initiated through altered cell division patterns in a small group of founder cells (referred to below as the preprimordium) on the flank of the meristem. The protrusion of these cells away from the meristem specifies the proximodistal axis of the primordium and the phyllotaxis of the plant. We propose that adaxial/abaxial polarity is imposed on this preprimordium, in a manner essential for the lateral recruitment of founder cells around the apex and for the subsequent patterning of adaxial and abaxial cell types (Fig. 7). Our data can not distinguish whether the ground state of the preprimordium is abaxial or whether the preprimordium has a non-defined ground state. In the latter case, both adaxial and

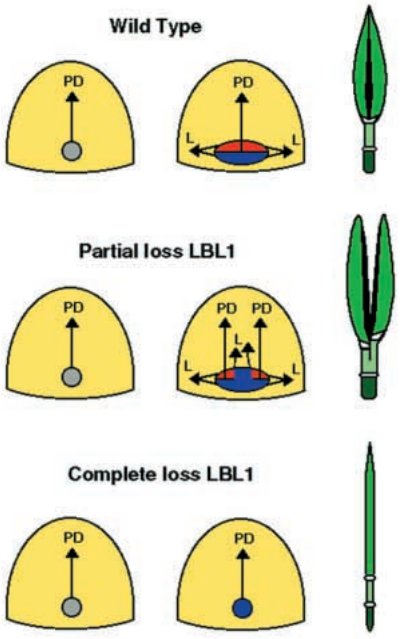

Fig. 7. Model for the action of the *Lbl1* gene product. See Discussion for an explanation of the model. The ground state of founder cells in the preprimordium is shown in gray. Cells in the adaxial and abaxial domains of the developing primordium are shown in red and blue, respectively. PD, points of proximodistal growth; L, points of lateral growth.

abaxial identities are imposed on the preprimordium, and the adaxial signal(s) must antagonize the abaxial signal(s), such that in the absence of adaxial patterning the lateral organ adopts an abaxial identity.

Lbl1 gene function is required to impose adaxial cell identity on the preprimordium. In the absence of LBL1 activity, founder cell recruitment stops, the primordium becomes radially symmetrical, and obtains or maintains an abaxial identity (Fig. 7). Reduced or patchy *Lbl1* expression could result in the various weak *lbl1* mutant phenotypes. For instance, leaves with half blade regions could result from LBL1 activity in just half of the adaxial domain of the preprimordium. Development of ectopic laminae or bifurcations near the midrib at the leaf tip could result from LBL1 activity in two patches on the sides of the preprimordium (Fig. 7). Cells in the center would obtain an abaxial identity, whereas the flanking regions establish adaxial/abaxial asymmetry and thus undergo lateral growth in both directions. Alternatively, a higher level of LBL1 activity may be required for adaxial patterning of the central region than for lateral growth of the primordium. This seems less likely, because gradual increases in LBL1 activity in radially symmetric *lbl1* leaves result in adaxial/abaxial asymmetry around the midvein without the formation of laminae. A third explanation for the development of bifurcated leaves is that two leaves are initiated simultaneously at adjacent positions. This is unlikely because the phyllotaxis of the most severely affected *lbl1* mutant plants remains distichous.

The formation of ectopic laminae versus bifurcations could depend on the proximity of the *Lbl1*-expressing patches in the preprimordium. If the patches are in close proximity, the central tissue could develop at the same rate and ectopic laminae would form. If the patches are further apart, the tissue between them develops more slowly (consistent with the reduced proximodistal growth of abaxialized, radially symmetrical *lbl1* leaves), and the primordium would bifurcate. Delayed accumulation of LBL1 in weak mutants would bring the *Lbl1*-expressing domains in closer proximity later in development and result in the formation of ectopic laminae proximal to the bifurcation. The length of the ectopic laminae and bifurcations would thus depend on the time during leaf development that LBL1 accumulation restores adaxial/abaxial patterning of the central region of the primordium. Depending on their length, ectopic laminae and bifurcations alter leaf patterning along the proximodistal axis or result in complete duplication of the organ. These observations are reminiscent of the observations made by Sachs (1969) following surgical experiments of leaf primordia in pea. Depending on the developmental stage of the primordium, incisions resulted either in duplication of the entire organ or in altered patterning of regions within the organ, suggesting that leaf primordia become gradually more patterned and determined.

Site of LBL1 action

The nature of the *lbl1* mutant phenotypes suggests that *Lbl1* acts on the preprimordium, at the site of insertion during further founder cell recruitment, and early in primordium growth and development. We can not distinguish whether *Lbl1* is also required during later stages of primordium development. Adaxial cell types may be determined early or adaxial identity may be maintained by downstream genes, possibly *Rld1* or *Ce1*. The *lbl1* mutant phenotype is more pronounced in the epidermis and only in radially symmetric *lbl1* leaves is adaxial/abaxial patterning of the vasculature affected. This suggests that epidermal and vascular tissues respond differently to reduced LBL1 levels, or that residual expression of *Lbl1* is higher near vascular bundles. Even though it is unclear whether LBL1 acts cell autonomously within a particular cell layer, the formation of ectopic laminae, bifurcations, and narrow leaves suggest a limited inductive range for LBL1. Similarly, the development of leaves with just half a blade region suggests that LBL1 acts autonomously on either side of the midvein/midrib. It is interesting to note that the deficient sides of *lbl1* half-leaves frequently give rise to thread-like outgrowths, suggesting that the subthreshold level of *Lbl1* occasionally permits additional proximodistal outgrowth.

Differentiation of leaf margins

Based on the *ns* mutant phenotype, P0 primordium development was suggested to proceed via two distinct steps (Scanlon et al., 1996). The initial step results in the recruitment of founder cells, whereas the later step specifies the lateral domains of the primordium. The *Ns* genes are suggested to initialize cells in the pre-marginal domain of the meristem to become founder cells. A failure to initialize these cells in the *ns* mutant would prevent these cells from responding to a lateral domain-specification signal and subsequently result in deletion of the leaf margins. Despite a defect in founder cell recruitment, *lbl1* mutant leaves develop normal margins with exception of the radially symmetric, thread-like leaves. Therefore, the *Ns* genes function not just in founder cell recruitment as suggested, but have a role in the differentiation

of marginal characters within specific lateral domains of the leaf. The presence of marginal characters on the narrow *lbl1* mutant leaves could thus result from altered expression of the *ns* genes in this mutant background. The *lbl1* phenotype further suggests that marginal characters develop at the boundary between adaxial and abaxial domains, and can develop in the absence of lateral growth.

Diversity of monocot leaf morphology

Monocot leaf morphologies range from almost entirely unifacial and radially symmetric (e.g. *Sansevieria suffruticosa*) to completely bilaterally asymmetric (e.g. maize). Many monocot species develop a bifacial, dorsoventral leaf blade with a short unifacial, radially symmetric tip, termed precursor tip or Vorläuferspitze (e.g. *Sansevieria trifasciata* and *Hosta lancifolia*) (see Kaplan, 1973). These leaf morphologies could result from differences in the expression patterns of *Lbl1* or other genes involved in specifying adaxial/abaxial identities. For instance, delayed expression of such genes would result in extended development of the radially symmetric, unifacial tip. This view that subtle differences in adaxial/abaxial patterning could be responsible for a broad variety of leaf morphologies is particularly attractive in light of the fact that development of a Vorläuferspitze evolved multiple times (Bharathan, 1996).

Leaf dorsoventrality and the shoot apex

Surgical experiments on shoot apical meristems of potato (Sussex, 1951, 1955), *Dryopteris* (Cutter, 1954; Wardlaw, 1949), *Epilobium* (Snow and Snow, 1959), and *Sesamum* (Hanawa, 1961) suggest that development of dorsoventral asymmetry in leaves requires a signal(s) from the shoot apex. Incisions that isolated the incipient leaf primordium from the apex resulted in the development of radially symmetric organs. Isolation of leaves which already displayed adaxial/abaxial asymmetry from the meristem did not result in the reversion of dorsoventrality to radial symmetry, suggesting that maintenance of adaxial/abaxial asymmetry occurs independent of the apex (Cutter, 1954; Sussex, 1955). Our observations on the *lbl1* mutant are consistent with these predictions.

A role for meristematic signals in the adaxial/abaxial axis specification allows the propagation of the direction of this axis in successive lateral organs. The nature of the meristematic signals required for adaxial/abaxial patterning of leaf primordia is unknown. Expression of *rs1* and *knox3* at the base of the disc of insertion suggests a possible role for these genes in specifying the abaxial domain of the incipient leaf (Jackson et al., 1994; Schneeberger et al., 1995). Several genes have also been identified that are expressed at the tip of the shoot apex (Clark et al., 1997; Pri-Hadash et al., 1992). *kn1* expression surrounds the P0 primordium so that *kn1* is not likely to play a role in adaxial/abaxial axis specification. Nonetheless, LBL1 plays a direct or indirect role in the downregulation of KN1 in leaf founder cells. In addition, knots resembling those induced by dominant *Kn1* alleles develop on *lbl1* leaves at the base of ectopic laminae and bifurcations. However, no KN1 accumulation was detected in radially symmetric *lbl1* leaf primordia. It is possible that KN1 interacts with other factors in the adaxial and/or abaxial domain of the leaf. The knots observed on dominant *Kn1* and weakly phenotypic *lbl1* mutant leaves develop the same specific orientation, with sheath/auricle tissue on the abaxial leaf surface. Also, ectopic expression of *KNAT1*, a *kn1*-related gene from *Arabidopsis*, results in the development of ectopic meristems only on the adaxial leaf surface (Chuck et al., 1996). This potential interaction between *kn1* and adaxial leaf characters could be important for the formation of knots only at the base of ectopic laminae and bifurcations.

Similarity of dorsoventral patterning of lateral organs in plants and animal

The *lbl1* mutant phenotypes illustrate several similarities between the development of lateral organs in plants and lateral appendages in *Drosophila*. *Drosophila* appendages develop from groups of initial cells, the imaginal discs, which are subdivided into specific domains by the expression of selector genes. For instance, expression of the homeodomain protein encoded by the *apterous* (*ap*) gene specifies the dorsal compartment of the wing disc (Cohen et al., 1992). *ap* gene function is required for the determination of dorsal cell types in the wing and for proliferation of the wing. The juxtaposition of *ap*-expressing and *ap*-nonexpressing cells specifies the position of the wing margin and results in proximodistal growth of the wing (Williams et al., 1993, 1994). Induction of ap^- clones in the dorsal compartment of the wing disc causes the formation of patches of ventral tissue on the dorsal wing surface that are surrounded by ectopic margins (Diaz-Benjumea and Cohen, 1993). Both *Lbl1* and *ap* are thus necessary for the specification of the adaxial/dorsal domain within the primordium, for the specification of the margin, and for the determination of adaxial/dorsal cell types. However, loss of *ap* gene function results in a loss of wing outgrowth, indicating that *ap* is required for the specification of the proximodistal axis (Butterworth and King, 1965; Cohen et al., 1992). In contrast, radially symmetric *lbl1* mutant leaves maintain the ability to grow out, suggesting that proximodistal growth can occur in the absence of adaxial/abaxial axis formation (see also Waites and Hudson, 1995). Nonetheless, the fixation of points of proximodistal growth during lateral organ development in plants may be dependent on the establishment of the adaxial/abaxial axis. Duplication of the adaxial/abaxial axis, perhaps because of patchy expression of *Lbl1* in the preprimordium, results in bifurcation of the primordium and specification of multiple points of proximodistal growth. The point of proximodistal growth within *Drosophila* imaginal discs appears to be specified at the intersection of the dorsoventral and anterior-posterior axes (Campbell and Tomlinson, 1995). A similar mechanism could operate in the fixation of points of proximodistal growth in lateral organs in plants.

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