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Xcl1 causes delayed oblique periclinal cell divisions in developing maize leaves, leading to cellular differentiation by lineage instead of position

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SUMMARY

Differentiation of plant cells is regulated by position-dependent mechanisms rather than lineage. The maize *Extra cell layers1* (*Xcl1*) mutation causes oblique, periclinal divisions to occur in the protoderm layer. These protodermal periclinal divisions occur at the expense of normal anticlinal divisions and cause the production of extra cell layers with epidermal characteristics, indicating that cells are differentiating according to lineage instead of position. Mutant kernels have several aleurone layers instead of one, indicating that *Xcl1* alters cell division orientation in cells that divide predominantly in the anticlinal plane. Dosage analysis of *Xcl1* reveals that the mutant phenotype is caused by overproduction of a normal gene product. This allows cells that have already received differentiation signals to continue to divide in aberrant planes and suggests that the timing of cell division determines differentiation. Cells that divide early and in the absence of differentiation signals use positional information, while cells that divide late after perceiving differentiation signals use lineage information instead of position.

Key words: *Xcl1*, Leaf, Maize, Differentiation, Cell position, Lineage, Multiple aleurone, Protoderm, Cell division orientation

INTRODUCTION

The development of plant form begins at the meristems, centers of undifferentiated cells at the shoot and root apices. The shoot apical meristem (SAM) is responsible for the production of lateral organs such as leaves through the orchestration of elaborate gene expression cascades. A fundamental question in plant development is how these undifferentiated cells divide and differentiate to form leaves and other lateral organs.

In higher plants the SAM can be divided into the tunica and the corpus. Monocot SAMs have one or two tunica layers; in maize there is only one tunica layer, the L1, and the corpus, the L2 (Esau, 1977). During development, cells can divide in anticlinal, periclinal, or random orientations. In anticlinal divisions, the new cell walls are inserted perpendicular to the surface of the organ, whereas in periclinal divisions the new cell walls are inserted parallel to the surface of the organ. Leaf clonal analysis experiments in maize have shown that the L1 layer gives rise to the protoderm that will form the epidermis through controlled anticlinal divisions. The mesophyll, bundle sheath, and vascular tissue originate from anticlinal and periclinal divisions in the L2 layer (Poethig, 1984).

Specific planes of cell division are required at certain stages of plant development. In *Arabidopsis* roots cortical/endodermal initials in the root apical meristem undergo periclinal divisions in order to form two distinct cell layers known as the outer cortex and the endodermis. The *scarecrow* mutation prevents this periclinal division, and mutant roots have only one cell layer with both cortical and endodermal features (Di Laurenzio et al., 1996). Periclinal divisions are also important in leaf initiation at the shoot apex (Lyndon, 1982). In normal maize leaf development, protoderm cells divide predominantly in the anticlinal plane with few exceptions. The ligule, a thin flap of tissue at the junction of blade and sheath, is initiated by periclinal divisions of protoderm cells at a defined stage in leaf development. In the margins of leaves the L1 layer can give rise to internal tissue by infrequent periclinal divisions (Poethig, 1984). Similar rare L1 derivative intrusions have been noted in clonal analysis experiments, and appear to occur before the mesophyll cells have differentiated (Dawe and Freeling, 1991). In all such reported instances, the cells that are formed as the result of a periclinal division from another layer acquire their tissue identity based on position and not lineage suggesting that position is more important than lineage in specifying ultimate cell fates.

Little information exists about how planes of cell division are controlled during plant development. The determination of new cell plate orientation involves decisions on whether a cell will divide in the periclinal plane or in the anticlinal plane. The well-organized patterns of cell division in the maize leaf have allowed the identification of mutations such as *tangled1* and *warty1* (Reynolds et al., 1998; Smith et al., 1996) that affect planes of cell division. The *Extra cell layers1* (*Xcl1*) mutation is a semi-dominant mutation that disrupts cell division and differentiation patterns in maize. In *Xcl1* mutants, normal

patterns of cell division in the developing maize leaf are altered, resulting in the formation of an extra epidermal layer through frequent periclinal (rather than anticlinal) divisions of the protoderm. Therefore, we propose that the *Xcl1* gene product is likely involved in the first stage of the determination of cell division plane and may be important in specifying whether cells divide anticlinally or periclinally. Since these L1-derived internal cells do not differentiate into mesophyll cells but retain epidermal features, this mutation may also shed light on the control of tissue differentiation in the maize leaf.

MATERIALS AND METHODS

Plant material

The *Xcl1* mutation arose in active *Mutator* transposon lines and was generously provided by Dr Patrick Schnable, Iowa State University. The mutation was introgressed five generations into W23 (with dominant color genes) and four generations into B73 and Tama Flint inbred backgrounds.

Histology

For general anatomical and immunolocalization studies, 1 cm pieces of tissue from the midpoint of the first seedling leaf from 2-week old seedlings were fixed in FAA, dehydrated through an ethanol series, and embedded in Paraplast+ (Oxford Labware). Sections were either stained with 0.05% Toluidine Blue O or were subjected to immunohistochemistry as previously described (Sinha and Lynch, 1998).

For plastic sections, serial pieces of tissue ranging from the stem to the tip of the coleoptile of newly germinated seedlings were fixed overnight at 4°C in 2.5% paraformaldehyde and 1% glutaraldehyde in phosphate buffer, pH 7, followed by infiltration with Historesin (Leica). Blocks were sectioned at 2.5 μm and stained using either the Periodic acid-Schiffs (PAS) reaction (Ruzin, 1999), or Feulgen's reaction for nuclear staining (De Tomasi, 1936) followed by the PAS reaction. Recent cell divisions were identified by paired nuclei separated by thin cell walls. Cell division orientations were classified as anticlinal or oblique anticlinal if the recent cross wall connected the two cell walls parallel to the organ surface, and periclinal or oblique periclinal if the recent cross wall connected the two cell walls perpendicular to the surface. Slides were viewed on a Nikon Eclipse E600 and images were captured using a SPOT RT-color CCD camera (Research Diagnostics, Inc.).

KN1 immunolocalizations

Immunolocalizations on meristems were performed according to published protocols (Smith et al., 1992) using polyclonal antibodies to KN1 generously provided by Dr Sarah Hake (USDA-PGEC in Albany, CA) or polyclonal antibodies prepared and affinity purified by Antibodies, Inc. (Davis, CA) using a bacterially expressed maize KN1 protein. The KN1 shoot expression detected by this antibody mirrored that of the antibody generated by Smith et al. (Smith et al., 1992).

in situ hybridization

In situ hybridizations were performed on 2-week old maize apices as previously described (Jackson et al., 1994; Long et al., 1996). The *ZmOCL5* probe was generously provided by Dr Peter Rogowsky, RCAP ENS, Lyon.

SEM of kernels

Kernels were left to imbibe for 1-2 days then cut in half and fixed in FAA overnight. Samples were then osmicated and critical-point dried in a Tousimis Samdri-780A. After coating the samples with 25-30 nm gold on a Denton Vacuum DeskII, samples were viewed with a Hitachi S-3500N scanning electron microscope at 5 kV accelerating voltage.

Clonal analysis

Xcl1 homozygotes and/or heterozygotes in the Full color/W23 background were crossed to *Xcl1* mutants in non-colored backgrounds (B73, F86). Kernels were soaked until the radicals emerged and then were irradiated with 1200 rads of gamma irradiation from a cesium source (ITEH, UC-Davis). Plants were grown in the greenhouse and examined for sheath sectors, which were free-hand sectioned into 50% glycerol to retard leaching of anthocyanins.

Epidermal cell division patterns

For analysis of epidermal cell division patterns, young leaves ranging in length from 0.2-2.5 cm and corresponding to P2-P4 leaf primordia were dissected from 2- to 3-week old seedlings. Cell division patterns were examined using the replica method (Sylvester et al., 1990).

Morphometric analyses

Xcl1 segregating families were grown in the field for measurements of adult leaves. Leaf blade length was determined by measuring from the ligule to the tip of the blade, and width and thickness measurements were taken at points a quarter, a half and three quarters the length of the blade. Leaf thickness was measured using a Plastical digital caliper (Forestry Products, Inc.). Data was analyzed using Microsoft Excel.

Dosage analysis

For dosage analysis, *Xcl1* homozygous ears, which were also homozygous for the 10L marker *r* were pollinated by pollen from TB-10L hyperploid stocks provided by the Maize Stock Center, Urbana-Champaign. Kernels were separated based on size (small kernels indicate hypoploid endosperm and hyperploid embryo) and color markers (kernels that had hypoploid endosperm were yellow owing to a lack of transmission of the dominant *R* allele from the male parent) (Beckett, 1994; Birchler, 1994) and grown to maturity in the greenhouse under standard conditions. At maturity, pollen abortion rates were determined by collecting pollen from fresh anthers and classifying the pollen grains under a dissecting microscope.

RESULTS

The *Xcl1* leaf phenotype

Xcl1 is a semi-dominant mutation which was identified by its effect on leaf morphology. In the juvenile phase of development, *xcl1* (wild-type) maize leaves have a glaucous appearance (Fig. 1A), while *Xcl1/Xcl1* leaves are thick and narrow and have an abnormally shiny appearance (Fig. 1B). Throughout development, the *Xcl1* phenotype is readily identified by the thickness of the leaves and concurrent reduction in leaf width. *Xcl1* was introgressed into three different genetic backgrounds (B73, W23, and Tama Flint) and segregated as a single semi-dominant locus in all backgrounds tested. Leaf blades of mature plants were measured for comparisons of length, width, and thickness between *Xcl1/Xcl1* and normal siblings (*Xcl1/xcl1* plants had an intermediate phenotype). In all backgrounds, *Xcl1* blades were slightly shorter than in normal siblings (Fig. 1C). This effect was most pronounced at the top of the plant, but the leaves followed the same growth trajectory as in normal siblings: short at the base of the plant, longer in the middle, and short again at the top of the plant. A more interesting relationship was seen when the width and thickness of *Xcl1* leaves was compared to the normal

sibling leaves. At three different positions along the leaf blade, *Xcl1* leaves were up to twice as thick as the normal sibling leaves (Fig. 1E), but as little as half as wide (Fig. 1D), indicating that the *Xcl1* mutation affects cell division and/or expansion in the width and thickness dimensions. The effects on width and thickness were most pronounced in the Tama Flint background, which has narrower leaves, a shorter time to flowering, and fewer leaves produced than the other inbred backgrounds investigated. In *Xcl1* leaf blades, width is decreased by a similar amount at all three positions measured, indicating that the reduction in width is consistent throughout the length of the blade, and therefore blade proportions (or shape) are not changed by the presence of the *Xcl1* mutation.

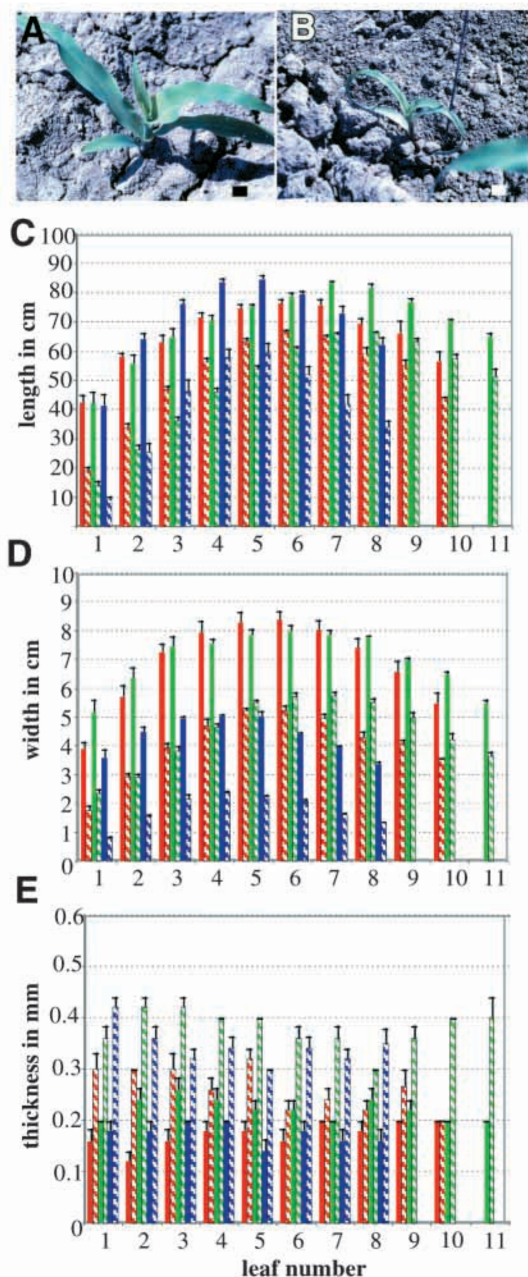

Fig. 1. Effects of *Xcl1* on leaf morphology. (A) Wild-type seedling. (B) *Xcl1* seedling has narrow, thick leaves that are shiny in appearance. Scale bars 2 cm. (C-E) Morphometric analysis of adult leaves (numbered from top of plant down) in the Tama Flint (Blue), W23 (Red) and B73 (green) backgrounds. Wild-type values are solid bars and *Xcl1* values are hatched. (C) In all backgrounds, *Xcl1* leaves are significantly shorter than wild-type leaves. (D) At the midpoint of the leaf blade, *Xcl1* leaves are half as wide as wild-type leaves. (E) At the midpoint of the leaf blade, *Xcl1* leaves are twice as thick as wild type. Similar width and thickness effects were seen at 1/4 and 3/4 the length of the blade.

The increased leaf thickness in *Xcl1* was caused by the production of large, vacuolated cells (similar to bulliform cells) between the mesophyll and the epidermis on both the adaxial and abaxial sides of the leaf blade, leading to extra cell layers in the blade (Fig. 2A,B). The spacing of major lateral veins and the arrangement and number of bundle sheath and mesophyll cells around veins were unchanged in *Xcl1* leaves. However, *Xcl1* leaves had a reduced number of second order veins between lateral veins suggesting that the defect manifests itself when these second order veins are being formed.

The differentiation of the epidermis was also affected in *Xcl1* mutant leaves. A normal maize epidermis has longitudinal rows of crenulated, rectangular intercostal cells, bulliform cells, and regularly spaced stomata (Fig. 2C,E) (Freeling and Lane, 1994). The epidermis of *Xcl1* mutant leaves was noticeably altered: pavement cells were square in shape and much less crenulated. Also, stomata were very infrequent and irregularly spaced. Mutant leaves had few to no stomata on the adaxial surface, and fewer stomata than normal on the abaxial surface (Fig. 2D,F). Thus, the semidominant *XclI* mutation caused a consistent decrease in leaf width and increase in leaf thickness accompanied by alterations in epidermal differentiation.

Effects of *Xcl1* on other parts of the plant

Even though the *Xcl1* mutant phenotype was most evident in leaf blades, effects on other parts of the maize plant were also seen. When examined by scanning electron microscopy, *Xcl1* meristems were shorter than the meristems of wild-type siblings. KNOTTED1 (KN1) is expressed in the SAM and is down-regulated in incipient leaf primordia (P_0) and developing leaves (Jackson et al., 1994; Smith et al., 1992). The accumulation of KN1 in *Xcl1* meristems appeared normal, but the apical dome was significantly shorter in mutant than in wild-type meristems (Fig. 3A,B). When the number of leaf founder cells were determined by counting nuclei in areas of KN1 downregulation in serial sections, *Xcl1* meristems had nearly twice the number of cells recruited into P_0. The severity of the *knotted1* loss-of-function phenotype is affected by meristem height of the inbred used for introgression (Vollbrecht et al., 2000). In each inbred background tested, *Xcl1* meristems were approximately two-thirds the height of wild-type sibling meristems (Table 1). When wild-type meristem heights were compared between the different inbred lines, Tama Flint meristems were significantly taller than B73 or W23 and the *Xcl1* phenotype was most severe in the Tama Flint background. Meristem width (Table 1) did not appear to correlate with the *Xcl1* phenotype.

When *Xcl1* and wild-type roots were examined, the most noticeable difference was that the *Xcl1* mutant has shorter zones of cell division and elongation with root hairs occurring very close to the root apical meristem and root cap, but there was no detectable effect on numbers of cell layers in transverse sections of the zone of maturation (Fig. 3C,D). The *Xcl1*

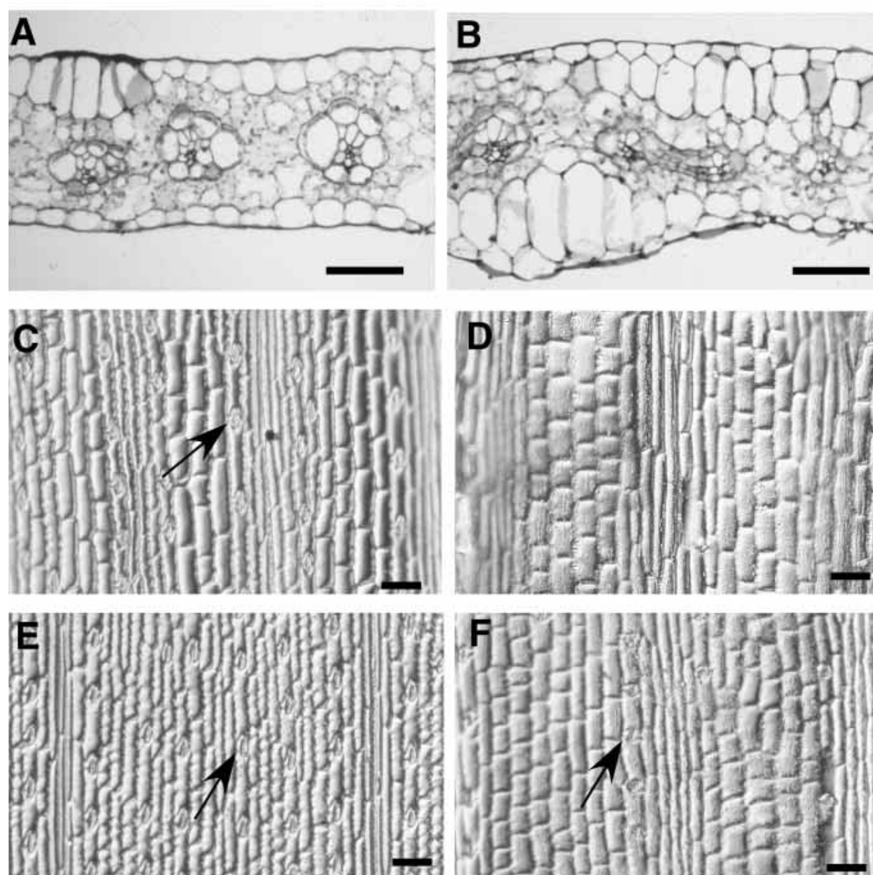

Fig. 2. Effects of *Xcl1* on leaf anatomy. (A) Transverse section of a wild-type adult leaf. (B) Transverse section of *Xcl1* leaf with large, thin walled cells beneath both the adaxial and abaxial epidermis. (C,E) Replica images of adaxial (C) and abaxial (E) epidermis of a wild-type leaf showing the regular placement of stomatal complexes (arrows) and crenulated pavement cell walls. (D,F) *Xcl1* adaxial (D) and abaxial (F) epidermis with reduced stomatal complexes and crenulation of pavement cell walls. Scale bars, 100 μm.

mutation also affects cell division patterns in the kernel. In *Xcl1* kernels, an extra aleurone-like layer was observed (Fig. 3F) in contrast to only one cell layer seen in wild type (Fig. 3E). The most prominent effect of the *XclI* mutation was the increase in cell layers in aerial plant parts and aleurone of kernels, but the reduction in meristem height indicates that *Xcl1* exerts its function very early in development.

Identity of the extra cell layers

For further analysis of the developmental effects of *Xcl1*, we focused on the homozygous *Xcl1* phenotype. The ZmOCL family of HDZIP genes are expressed in the protoderm of leaves and other organs (Ingram et al., 2000). ZmOCL5 mRNA was detected only in the outer cell layer of meristems and young leaves in both wild-type and *Xcl1* individuals examined (Fig. 4I-J). The extra cell layers in the *Xcl1* mutant have large vacuoles, thin cell walls, and lack chloroplasts, therefore, they appear to resemble epidermal cells instead of any of the internal cell types. In order to determine the biochemical identity of the extra cell layers in *Xcl1* mutant leaf blades, immunolocalizations were performed using cell-type-specific antibodies to distinguish between bundle sheath, mesophyll and epidermal cells (Sinha and Hake, 1994; Sinha and Lynch, 1998). Ribulose bisphosphate carboxylase/oxidase (RuBisCO) is a marker for bundle sheath cells in C4 plants, while phosphoenolpyruvate carboxylase (PEPC) is a marker for mesophyll cells (Langdale et al., 1987; Langdale et al., 1988). Neither antibodies to the small subunit of RuBisCO (Fig. 4A,B) nor antibodies to PEPC (Fig. 4C,D) labeled the extra cell layers in cross sections of *Xcl1* mutant leaf blades, indicating that these extra cells are not functional mesophyll or bundle sheath cells. A monoclonal antibody to β (1,3) and (1,4) mixed-linkage glucans cross-reacts with epidermal and vascular cell walls in wild-type maize leaves (Fig. 4E) (Sinha and Lynch, 1998). This antibody also labeled the extra cell layers in the *Xcl1* mutant (Fig. 4F), indicating that the extra cells resemble epidermal cells. Anthocyanin production is limited to the epidermis in the maize blade (Fig. 4G) (Freeling and Lane, 1994), and is therefore a useful marker for epidermal cell identity. In *Xcl1* leaf blades, the extra cells produced anthocyanins at levels comparable to that found in wild-type epidermal cells (Fig. 4H). These results indicate that the extra cell layers are more similar to epidermal cells than the normal internal tissues of a maize blade.

Clonal analysis to determine origin of extra cell layers

The anatomy of *Xcl1* leaves suggests that the extra cell layers may have one of two origins: aberrant periclinal divisions in the L2 leading to cells that differentiated into epidermal-like cells instead of mesophyll, or aberrant periclinal divisions of the protoderm with maintenance of epidermal identity in the daughter cells. We performed a clonal analysis using anthocyanin markers in order to determine the lineage of extra cells in the *Xcl1* leaf. In maize, anthocyanin production is dependent on the presence of the dominant alleles of several color genes (Coe, 1988). In contrast to the maize leaf blade, which produces anthocyanins only in the epidermis (Freeling and Lane, 1994), the leaf sheath can produce anthocyanins throughout all tissue layers, with more in the layers closer to the surface (Fig. 5B-C). *Xcl1* plants heterozygous for the factors necessary for anthocyanin production were generated to facilitate a clonal analysis of the extra cell layers. Seedlings were irradiated to induce chromosomal breaks which would cause loss of dominant color alleles in some cells, leading to colorless sectors (Fig. 5A).

In the wild-type sheath, second order veins were appressed against the abaxial epidermis, while the *Xcl1* sheath had 3-4 extra layers of cells between secondary veins and the abaxial epidermis (Fig. 5B,C). The majority of sectors identified extended through both the epidermis and all internal cell layers

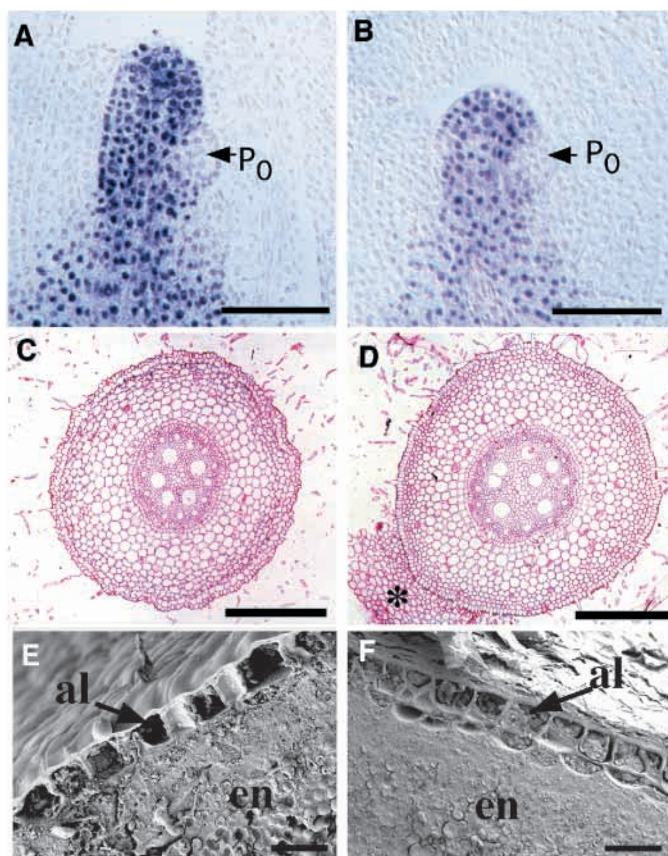

Fig. 3. *Xcl1* effects on other parts of the maize plant. (A) KN1 immunolocalization on a median longitudinal section of a wild-type meristem showing down-regulation in the P_0 and developing leaves. (B) KN1 expression in the *Xcl1* meristem is normal, but the apical dome is significantly shorter. (C,D) Transverse sections through the root hair zone of wild-type (C) and *Xcl1* (D) roots. The roots have similar numbers of cell layers (*indicates root cap cells). (E) SEM of wild-type kernel with pericarp removed showing one layer of aleurone cells (al) bordering the starchy endosperm (en). (F) *Xcl1* kernel has 2 layers of aleurone cells. Scale bars A-B, 100 μm; C-D 500 μm; E-F, 25 μm.

(Fig. 5D) and were therefore not useful in determining the clonal origin of the extra cell layers. However, two periclinal sectors were identified in which the epidermis had a different color status than the internal cell layers. In the first sector, the epidermis and extra cell layers expressed anthocyanins, while the internal layers were colorless (Fig. 5E). In the second sector, the epidermis and extra cell layers were colorless, but the internal layers expressed anthocyanins (Fig. 5F). Both of these results indicate that the extra cell layers in the *Xcl1* leaf sheath share a common origin with the epidermis and likely arose from aberrant periclinal divisions in the protoderm.

Developmental anatomy of *Xcl1* leaves

In order to determine the timing and location of the aberrant cell divisions in *Xcl1*, we analyzed thin serial transverse sections through the SAM for cell division patterns at various stages of early leaf development. As early as Plastochron 1 (P_1), the *Xcl1* leaf primordium was thicker than wild type. In later stages of development, the developing midrib region was much thicker in *Xcl1* than in wild type (Fig. 6A,B). However, the most pronounced differences between wild type and *Xcl1* leaves occurred at the developing leaf margins. During normal development, the P_1 leaf primordium has a three cell layer margin that continues to extend in order to accommodate the rapidly thickening stem as differentiation proceeds from the midrib outward (Sharman, 1942). A five-cell layer wide margin is characteristic of wild-type leaves at P_2 and P_3 (Fig. 6A). The *Xcl1* P_3 margin did not display the typical five-layered anatomy (Fig. 6B), instead margins appeared to be shortened, often had disorganized cell layers, and veins were initiated closer to the edge of the leaf.

Table 1. Average size of apical dome above P_0

Inbred background	Wild-type height	*Xcl1* height	*Xcl1*% of wild-type	Wild-type width	*Xcl1* width
Tama Flint	95±5.6	61±4.7	65	89±0.5	109±5.9
W23 (full color)	74±7.4	50±1.7	68	88±3.6	83±8.8
B73	69±6.1	46±0.2	66	82±0.3	69±0.8

Values are in μm±s.e.m. for different inbred backgrounds; *n*=3 for each class.

We examined serial sections of the margin in order to identify periclinal divisions in the protoderm of *Xcl1* leaves. When P4 primordia were examined at points ~200 μm above the tip of the SAM (or the midpoint of a 1 cm leaf), the *Xcl1* margin was much thicker and more disorganized than the wild-type leaf margin (Fig. 6B,F). Serial sections of the wild-type margin revealed that at this stage of development, recent cell divisions (identified by thin cross walls) in the protoderm are rare and occur only in the anticlinal plane (Fig. 6B-D). When packets of cells in the *Xcl1* margin were followed through serial sections, it was evident that cell divisions in the protoderm were not restricted to the anticlinal plane. The protoderm cells in *Xcl1* were larger than normal, and new cell walls and paired nuclei indicated that oblique periclinal divisions had occurred to produce additional inner cell layers from the protoderm (Fig. 6F-H). At the same stage of wild-type development, protodermal divisions in the anticlinal plane were very infrequent and periclinal divisions were not observed (Fig. 6B-D). Extra cell layer files could be seen converging with the epidermis at regions closer to the midrib (Fig. 6G) indicating that late periclinal divisions in the protoderm gave rise to the extra cell layers.

In order to determine the effects of the *Xcl1* mutation on epidermal development, we examined epidermal cell division patterns at different stages of leaf development. Anticlinal transverse divisions increase leaf length, while anticlinal longitudinal divisions increase leaf width. In the wild-type leaf protoderm, transverse divisions occurred throughout the 0.2 cm immature leaf then became gradually restricted to the base as the leaf grew to 2.5 cm (Table 2A). In *Xcl1* leaves, a reduction in the normal number of transverse divisions was first seen in the 0.5 cm leaf at the middle and tip regions of the blade, while divisions at the base of the blade were similar in *Xcl1* and wild type (Table 2A). By the 1 cm stage, cells at the tip of the blade had begun to differentiate and mature stomata were present. At this stage, transverse divisions were the same at the tip of the *Xcl1* and wild-type leaves, but were reduced at the middle and basal regions of the *Xcl1* blade. Thus, the reduction in anticlinal

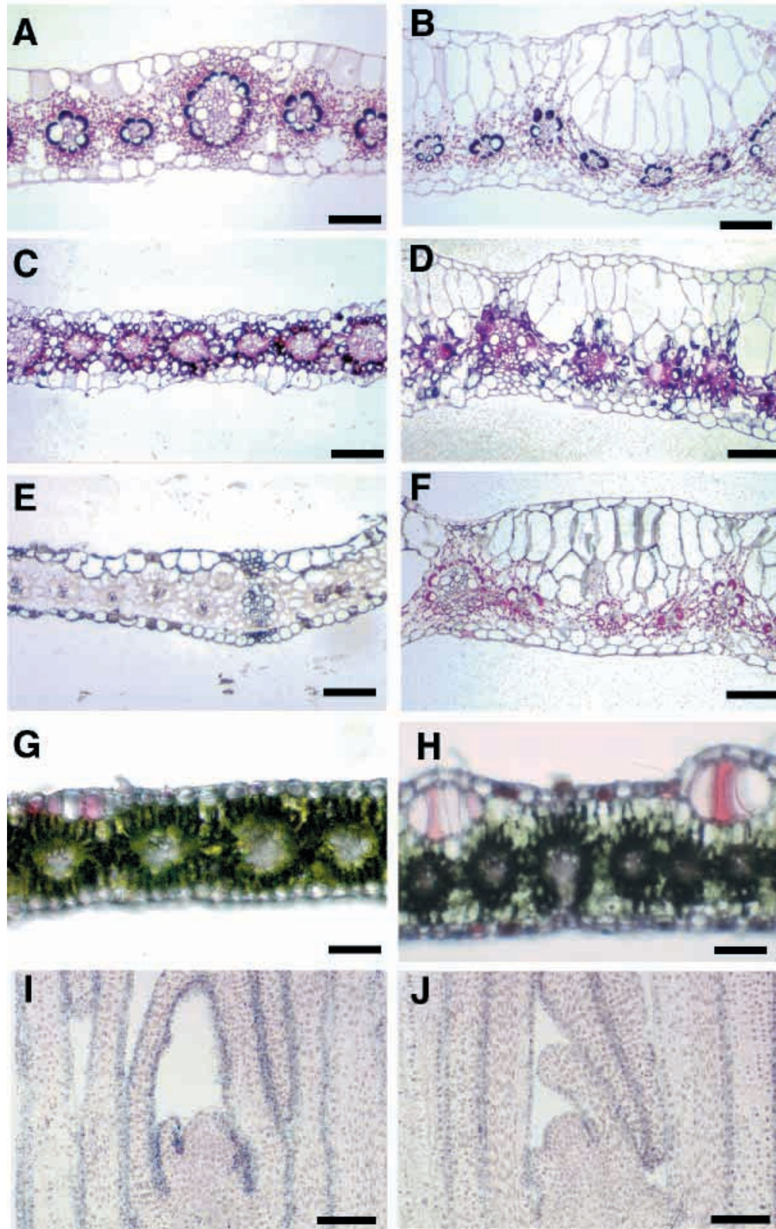

Fig. 4. The extra cells in the *Xcl1* mutant have epidermal identity. (A-F) Immunolocalizations with cell-type-specific antibodies. Silver enhancement of the gold-conjugated secondary antibody produced a black signal. (A,B) The small subunit of RuBisCO is localized to the bundle sheath cells in both wild-type (A) and *Xcl1* (B) leaves, but is not detected in the extra cell layer. (C,D) PEPC is localized to the mesophyll cells in wild-type (C) and *Xcl1* (D) leaves, but is not in the extra cell layer. (E-F) β (1,3) and (1,4) mixed linkage glucans are localized to epidermal and vascular cell walls in wild-type leaves (E) and in the extra cell layers in the *Xcl1* mutant (F). (G) Anthocyanin production is limited to the epidermis in the adult wild-type leaf. (H) Anthocyanins are produced in both the epidermis and the extra cell layers in *Xcl1*. (I-J) *ZmOCL5* is expressed in the outer cell layer in both wild-type (I) and *Xcl1* (J) meristems and leaves. Scale bars A-J, 100 μm.

transverse divisions in the *Xcl1* protoderm occurred in the same region of the P4 primordia where periclinal divisions were observed in serial leaf sections (Fig. 6F-H). This reduction is correlated with the progression of differentiation in the leaf blade. When longitudinal divisions were compared between wild-type and *Xcl1* leaves at the same stages of development (Table 2B), *Xcl1* had a slight increase in the number of longitudinal divisions at the base of 0.5 cm leaves and middle of 1 cm leaves. However, the main effect of *Xcl1* on epidermal development seems to be the conversion of transverse anticlinal divisions to oblique periclinal divisions.

Dosage analysis

The semidominant *Xcl1* phenotype of aberrant periclinal divisions in the protoderm could be caused either by a reduction in a protein that would normally repress periclinal divisions in the protoderm (hypomorph or antimorph) or by an increase or novel expression of a factor that promotes periclinal division (hypermorph or neomorph). In order to distinguish between these possibilities, a dosage series was generated using B-A translocation stocks (Birchler, 1994).

The *Xcl1* mutation maps to the long arm of chromosome 10 between the markers umc64 and umc44a. For dosage analysis, kernels from an *Xcl1*/TB-10L cross were classified based on kernel size and color (Fig. 7A,B). Plants were grown to maturity so that dosage could also be confirmed by rates of pollen abortion. Pollen abortion rates were as expected (Beckett, 1994), approximately 5-10% for diploids, 25% for hyperploids, and 50% for hypoploids.

The severity of the *Xcl1* leaf phenotype was compared in the dosage series. Hypoploid plants, which contained only the mutant *Xcl1* allele, had no extra cell layers in the leaf (Fig. 7E) and the leaf was not as thick as a wild-type diploid leaf (Fig. 7F). Hyperploid leaves, which contained two wild-type copies in addition to the mutant allele, had a more severe *Xcl1* phenotype than *Xcl1* heterogygous leaves (Fig. 7G,H), but a less severe phenotype than homozygous *Xcl1* leaves (Fig. 7I). Exacerbation of the *Xcl1* phenotype by the addition of wild-type gene doses suggests that *Xcl1* is a hypermorphic mutation that causes the overproduction of the *xcl1* gene product.

The phenotypes of B-A translocation lines are known to result from changing the dosage of whole chromosome arms. TB-10L hypoploids have been reported to have thin leaves that are wider than normal, while hyperploids have narrow, erect leaves (Neuffer et al., 1997). Dosage at the *Xcl1* locus may be one factor responsible for these plant phenotypes in the translocation stocks. While no extra cell layers were detected in the *xcl1* hypoploid and hyperploid leaves, hypoploid leaves had nearly twice the number of stomata per unit area than the hyperploid leaves (data not shown), indicating more anticlinal divisions in the protoderm. Kernels with *xcl1/xcl1/0* endosperm had a single layer of aleurone (Fig. 7C), while kernels with *xcl1/xcl1/xcl1/xcl1* endosperm had several patches of extra aleurone layers around the perimeter (Fig. 7D) similar to that seen in the *Xcl1* mutant (Fig. 3F), suggesting that increasing

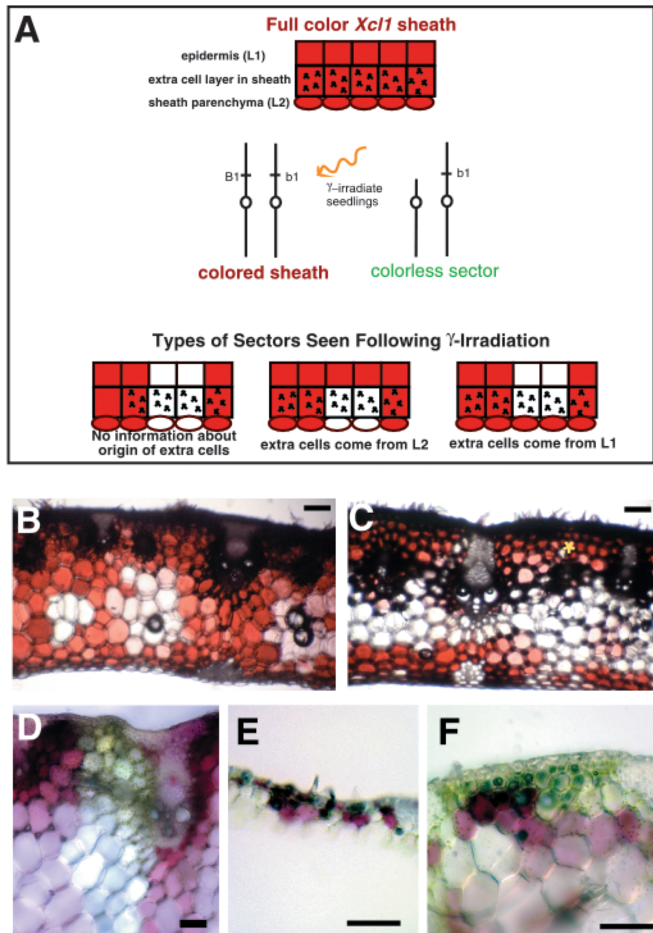

Fig. 5. Clonal analysis for determining the origin of the extra cells. (A) Experimental design. (B) Wild-type sheath, expressing anthocyanins. (C) *Xcl1* sheath has extra cell layers (asterisk) between the minor veins and the adaxial epidermis. (D) Non-informative sector extends through the epidermis and internal cell layers. (E) Sector with colored epidermis and extra cells and colorless internal cells. (F) Colorless sector extending through epidermis and the extra cell layers. Scale bars B-F, 100 μm.

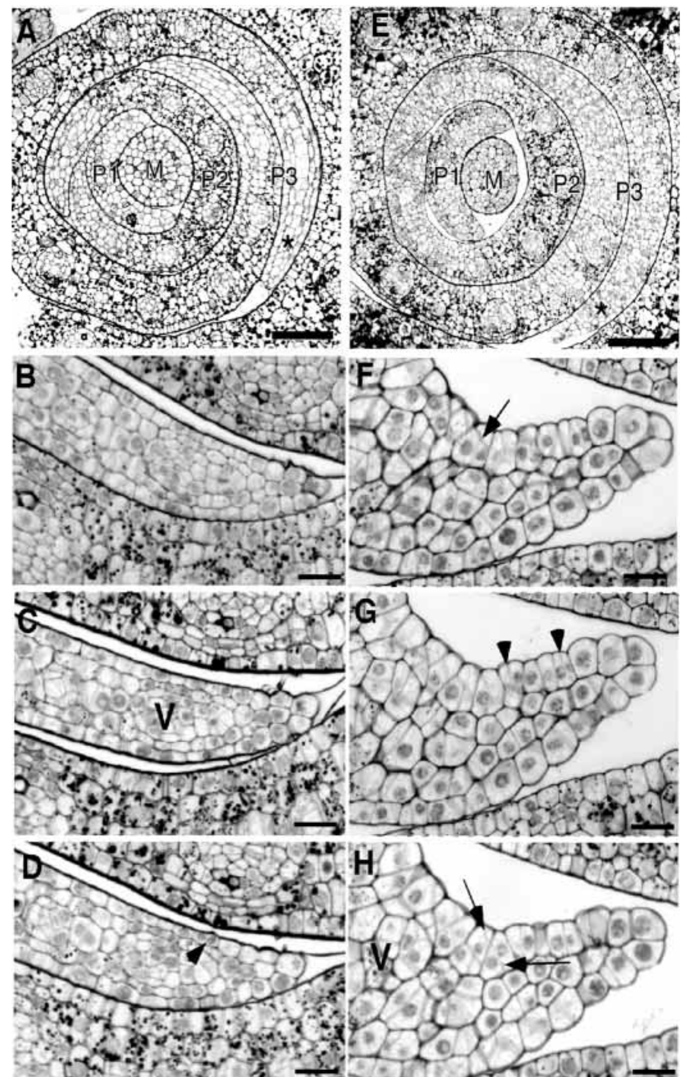

Fig. 6. Cell division patterns in developing leaf primordia. (A,E) Transverse sections through seedling meristems (M) including plastochrons (P) 1-3. (A) The wild-type leaves have 5 cell layer margins at P2 and P3 (asterisk). (E) *Xcl1* P_2 and P_3 margins have disorganized cell layers and are thicker than normal. (B-D) Serial sections through a wild-type P_4 margin with protoderm divisions (arrowhead) in the anticlinal plane and vascular bundles (V) near margin. (F-G) Serial sections through an *Xcl1* P_4 margin showing oblique periclinal divisions (arrows) in the protoderm and normal anticlinal divisions (arrowheads). Scale bars A, E, 100 μm; B-D and F-H, 25 μm.

the dosage of the wild-type *xcl1* allele can partially replicate the *Xcl1* phenotype.

DISCUSSION

The development of multicellular organisms from single-celled zygotes requires the coordination of cell division and differentiation processes. In animals, cell lineage has been shown to be important for specifying cell fate. Cell divisions and differentiation are so well conserved in *Caenorhabditis elegans* that the fate of cells can be predicted by examining cell division patterns (Sulston et al., 1983). In *Drosophila* development, early distribution of differentiation signals provides positional cues leading to lineage-dependent differentiation (reviewed by Lawrence and Struhl, 1996). In contrast, it has long been noted that plant cells acquire their differentiation signals based on their position relative to other cells and that lineage plays a minor role in the differentiation process (Poethig, 1984). The *Extra cell layers1* (*Xcl1*) mutant shows cell division and differentiation defects throughout development. Extra cells arise from oblique, periclinal divisions in the protoderm, but differentiate according to lineage and not position. A similar defect was seen in the aleurone of the kernel. Thus *Xcl1* provides a unique opportunity for examining the coordination of cell division and differentiation during plant development.

Meristem effects of *Xcl1*

Maintaining meristem size during development is critical for ensuring that the initiation of lateral organs proceeds normally.

Table 2. Recent divisions in the epidermis of developing leaves calculated as a percentage of the total number of cells in the region analyzed

	% divisions in wild-type: % divisions in *Xcl1*		
Leaf length	Base	Middle	Tip
A. Recent transverse divisions			
0.2 cm	7:10 (*n*=3)	8:5 (*n*=3)	8:7 (*n*=3)
0.5 cm	15:14 (*n*=6)	13:7* (*n*=8)	13:5* (*n*=6)
1.0 cm	12:7* (*n*=9)	13:6* (*n*=8)	5:6 (*n*=4)
2.5 cm	9:5* (*n*=7)	3:8* (*n*=6)	2:2 (*n*=5)
B. Recent longitudinal divisions			
0.2 cm	6:5 (*n*=3)	7:49 (*n*=3)	3:5 (*n*=3)
0.5 cm	4:8* (*n*=6)	2:3 (*n*=8)	0:1 (*n*=6)
1.0 cm	6:5 (*n*=9)	2:4* (*n*=9)	0.2:0 (*n*=4)
2.5 cm	0.4:0.2 (*n*=6)	0.2:0 (*n*=6)	0:0 (*n*=5)

*Pairs were significantly different by Student's *t*-test (*P*=0.0005). Standard errors were less than 1.7 in each instance. An average of 142 cells at the base, 101 cells in the middle, and 88 cells in the tip were scored for each sample.

Loss-of-function mutations in the *SHOOT MERISTEMLESS1* gene in *Arabidopsis* inhibit SAM establishment, maintenance, and subsequent lateral organ formation (Barton and Poethig, 1993; Long et al., 1996), while mutations in the *CLAVATA* gene and the overexpression of *WUSCHEL* increase meristem size and the number of lateral organs (Clark et al., 1993; Clark et al., 1995; Schoof et al., 2000). The *Xcl1* mutation also affects SAM size. In all of the inbred backgrounds tested, the number of founder cells recruited into the leaf primordium is larger in *Xcl1* than in wild-type and the leaf primordia are thicker from inception. There is a concomitant reduction (by approximately two-thirds) in the height of apical dome of *Xcl1* meristems when compared to wild-type sibling meristems. Anticlinal divisions in the L1 are necessary to expand the surface of the meristem and allow the meristem to be taller. The *Xcl1* gene could reduce meristem height either by promoting periclinal divisions in the L1 of the SAM at the expense of anticlinal divisions, or by downregulating *KNOTTED1-like* homeobox genes in the meristem and causing more cells to be allocated into leaf primordia.

Timing and location of aberrant cell divisions

In the maize *narrow sheath* mutant, leaves do not produce a marginal domain due to a failure to recruit a group of cells from the meristem into the leaf primordium (Scanlon et al., 1996). The resultant leaves are narrower than normal, indicating that the marginal domain of the leaf is critical in the development of normal width leaves. After leaf initiation, divisions at the margins expand the primordium to encircle the SAM, and the leaf begins to form veins and differentiate from the midrib out (Sharman, 1942). Characteristic regions of the margin (i.e. the 5-layered stage) were altered in the *Xcl1* primordia. Cells very close to the margin showed an increased number of divisions, especially oblique periclinal divisions in the protoderm leading to disorganized cell layers. However, in contrast to mutants like *tan1* that continue to be disorganized during tissue differentiation (Smith et al., 1996), in *Xcl1* the internal tissues differentiate normally but the epidermal layer is more than one cell thick and abnormal. The second epidermal layer cell files are offset from the outer epidermal files (Fig. 2B), highly suggestive of organized oblique periclinal divisions, in contrast to mutations like *warty*, where randomly oriented cell division lead to the formation of clusters of cells. The location and timing of aberrant periclinal divisions correspond with the decrease in transverse anticlinal divisions in the 1 cm leaf. Thus, in *Xcl1* mutants, some of the 'allocated' anticlinal marginal divisions in the protoderm seem to be converted to periclinal divisions at the stage when differentiation has begun, leading to thick, narrow leaves with a multi-layered epidermis.

Epidermal differentiation

As leaf development proceeds, protoderm cells differentiate into epidermal cells with interlocking walls, stomatal complexes and trichomes. While the main effect of the *Xcl1* mutation is the production of an extra layer of epidermal cells, the mutation also reduces the degree of crenulation in epidermal pavement cells. Normally, crenulations are seen in blade epidermal cells but not in the sheath epidermal cells, and juvenile epidermal cells are less crenulated than adult epidermal cells (Freeling and Lane, 1994). The smooth cell walls in the *Xcl1* mutant could be a result of changes in cell wall material distribution or cytoskeletal organization as cell differentiation proceeds. The reduced crenulations may also

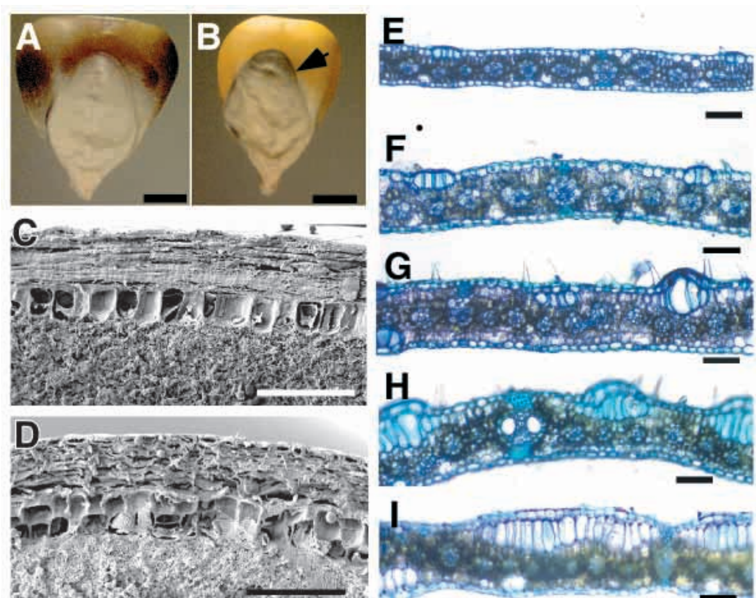

Fig. 7. Dosage analysis of *Xcl1*. (A) Large, colored kernel with hyperploid endosperm and hypoploid embryo. (B) Small, yellow kernel with hypoploid endosperm and hyperploid embryo with a colored scutellum (arrow). (C) TB-10L kernel hypoploid for *xcl1* has one layer of aleurone. (D) TB-10L kernel hyperploid for *xcl1* has regions with more than one layer of aleurone. (E-I) Free-hand transverse sections of leaves. (E) The hypoploid (*Xcl1/0*) leaf is thinner than wild-type and has no extra cell layers beneath the epidermis. (F) A wild-type (*xcl1/xcl1*) leaf. (G) Diploid (*Xcl1/xcl1*) leaf has infrequent patches of extra cell layers. (H) Hyperploid (*Xcl1/xcl1/xcl1*) leaf has a more severe extra cell layer phenotype than the diploid. (I) Homozygous diploid (*Xcl1/Xcl1*) leaf has the most severe extra cell phenotype. Scale bars A,B, 2 mm; C,I, 100 µm.

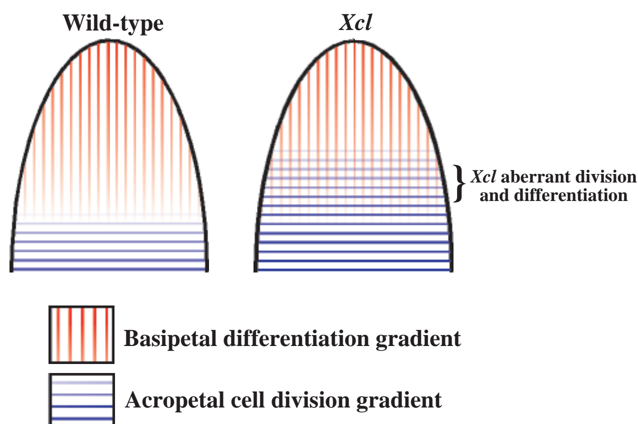

Fig. 8. Model for the lineage-dependent differentiation of the extra cell layers produced by aberrant periclinal divisions. In wild-type leaf primordia, an acropetal gradient of cell division does not overlap with the basipetal cell differentiation gradient, allowing cells to perceive positional information for proper differentiation. In the *Xcl1* mutant, the gradient of cell division competence is shifted up (possibly through the overproduction of *Xcl1* gene product), allowing cell divisions to occur after differentiation signals have been perceived.

represent a delay in the timing of differentiation of epidermal cells in the *Xcl1* blade such that these now resemble juvenile leaf blade or sheath cells.

Guard cell formation in maize requires an initial transverse anticlinal division to form the guard cell mother cell, followed by subsequent longitudinal divisions to form guard cells and subsidiary cells (Sharman, 1942). In dicots, lineage has been implicated in stomatal patterning (Larkin et al., 1997). In monocots position seems to be more important for specifying the linear arrangement of stomata (Hernandez et al., 1999), although mechanisms that provide the positional information have not been elucidated. *Xcl1* leaves have a reduction in the number of stomatal complexes formed. The predictable alignment of stomata in the epidermis with respect to internal tissue in the maize leaf indicates that internal signals might play a role in positioning the guard mother cells (Sylvester et al., 1996). Our analysis of protoderm cell division orientations in the anticlinal plane indicates a reduction in the number of transverse divisions in the *Xcl1* mutant at the stage of guard mother cell formation, possibly due to delayed or reduced signal perception in the protoderm caused by the extra cell layers beneath the epidermis.

Differentiation by lineage

In normal development, occasional periclinal divisions in the L1 at leaf margins produce internal cells that differentiate into mesophyll according to positional cues (Szymkowiak and Sussex, 1996). In roots, cortical initial cells that have been laser ablated are replaced by neighboring cells of different lineages (van Den Berg et al., 1997). Therefore, in plants, positional information usually dictates cell differentiation. The *ZmOCL* genes are expressed in the outer layers of organs and were proposed to be markers for protoderm cells (Ingram et al., 2000; Ingram et al., 1999). The *ZmOCL5* gene was expressed only in the outer cell layer and not in the extra cell layers in *Xcl1* meristems and developing leaves. These extra cell layers may be only partially specified as epidermal cells and therefore do not express all epidermal markers. However, our results with cell-type specific markers showed that the extra cells arising from oblique periclinal divisions in the protoderm of *Xcl1* have epidermal features, suggesting that ZmOCL5 may not be expressed in all cells with a protoderm lineage and may instead be a marker for cells that are on the outside of developing organs. The fate of the extra cell layers in *Xcl1* may be related to the timing of the aberrant periclinal cell divisions.

Model for Xcl1 function

Gradients have been shown to be important for specifying developmental patterns. Early in *Drosophila* development, morphogen gradients divide the embryo into compartments which will give rise to all body parts (Lawrence and Struhl, 1996). Likewise, in developing maize leaf primordia, a gradient of differentiation is present from the tip down, and a gradient of competence for cell division is present from the base of the leaf up. Freeling (Freeling, 1992) proposed that cells in a leaf primordium pass through various states of competency (from sheath to ligule/auricle to blade) to become different organ/tissue types. Mosaic analysis experiments with the *Liguleless3* mutant suggested that mutant cells did indeed differentiate according to their competency states (Muehlbauer et al., 1997). In normal leaf development, cells that have started to differentiate lose their competence for cell division. The effects of the *Xcl1* mutation on cell division and differentiation can best be explained by postulating that the gradients shift, leading to an overlap in the cell differentiation and division states (Fig. 8). The change in the gradients could involve a downward shift in the differentiation gradient and/or an upward shift in the cell division gradient. The location and timing of the aberrant periclinal divisions in *Xcl1* suggest that the mutation causes an upward shift in the cell division gradient, while *Xcl1* root anatomy suggests a downward shift in the differentiation gradient. This overlap of gradients allows protoderm cells in the differentiation zone to divide and differentiate according to lineage and not position. This model can be tested by looking for alterations in cell division patterns in the overlap region.

Dosage analysis of *Xcl1* indicated that it is a hypermorphic mutation. Since an *Xcl1*-like phenotype was also seen in endosperm hyperploid for wild-type TB-10L, it is likely that the *Xcl1* mutation is caused by changes in regulatory elements which lead to the overproduction of a normal gene product. TB-10L hypoploid phenotypes give us clues about the effects of reduced XCL1 activity. TB-10L hypoploid leaves are wider than diploid leaves (Neuffer et al., 1997) and have an increased stomatal frequency, suggesting that reducing XCL1 activity leads to more cell divisions in the anticlinal plane. Therefore, the *Xcl1* gene product is a dosage sensitive factor that not only balances periclinal versus anticlinal divisions, but is also a key player in setting up the gradient for cell division competence by directly or indirectly regulating the cell cycle. Increasing XCL1 may also shift the division gradient upward and allow partially differentiated cells to divide in the periclinal plane and differentiate according to lineage and not position (Fig. 8). Recently, Jankowski et al. (Jankowski et al., 2001) reported that in the *tangled1* mutation, bundle sheath cells that divide

aberrantly after they have started differentiation programs produce daughter cells that differentiate according to lineage and not position. These results support our hypothesis that overlapping cell division and differentiation competencies allow for differentiation to proceed by lineage-based mechanisms.

The increased size of protoderm cells in *Xcl1* leaves suggests that perhaps XCL1 overproduction leads to increased cell expansion in the periclinal dimension. Some hints that cell size may regulate the frequency of periclinal divisions are provided from reports that TB10L hypoploid leaves are thinner (less periclinal division and/or expansion) and wider than diploid leaves (Neuffer et al., 1997) (Fig. 7). Thus a viable alternative hypothesis for XCL1 function could be that XCL1 is involved in cell expansion in the periclinal dimension leading to more periclinal divisions.

In root laser ablation studies, van den Berg et al. (van den Berg et al., 1997) showed that positional information is contained in adjacent cells, and that the ablation of several neighboring cells reduced the signals required for postion-dependent differentiation. An alternative hypothesis for the failure of the extra cell layers in *Xcl1* to differentiate as internal cell layers could be that the extra cell layers form a self-sufficient unit which overcomes the positional information from surrounding mesophyll cells. However, in heterozygous *Xcl1* mutants, very small patches of extra cells (as few as one cell) do not differentiate as mesophyll cells suggesting that it is not the size of the extra cell layer containing unit that allows lineage dependent differentiation. The *Xcl1* phenotype indicates that the XCL1 protein is involved in controlling both the timing and orientation of cell divisions. The maize *warty1* mutation also leads to aberrant periclinal cell divisions in the protoderm which produce wart-like outgrowths on the surface of the leaf. Protoderm cell size is not regulated properly during the cell cycle, and cell division appears to be triggered when the cells exceed a critical size (Reynolds et al., 1998). *Xcl1* also affects protoderm cell size, and the large protoderm cells show a preponderance of oblique periclinal divisions. However, double mutants between *Xcl1* and *warty* show an additive phenotype (S. K. and N. S., unpublished data) indicating that *Xcl1* and *wty* are not in the same pathway. The *tangled1* mutation in maize causes new cell walls throughout the leaf to be inserted at oblique angles rather than right angles (Smith et al., 1996). *TANGLED1* encodes a microtubule binding protein which controls phragmoplast placement during cell division (Cleary and Smith, 1998; Smith et al., 2001). In *Arabidopsis*, the *SCARECROW* and *SHORT ROOT* genes encode transcription factors (Di Laurenzio et al., 1996; Helariutta et al., 2000) that affect periclinal cell divisions in the root apical meristem and starch-containing shoot hypodermis, but there is no effect on the epidermis of the plant (Wysocka-Diller et al., 2000). It is possible that a SCR/SHR-like pathway exists in protoderm derivatives and *XCL1* is one component of this pathway. Alternatively, *XCL1* may be a component of an as yet undescribed pathway which balances anticlinal and periclinal divisions in the plant.

Effects of Xcl1 on aleurone development

Following double fertilization of the egg cell and central cell, the 3N endosperm undergoes several rounds of free nuclear division to form a syncytium. Next, nuclei migrate to the periphery of the endosperm, start cell wall deposition, and divide periclinally. The inner daughter cell becomes part of the starchy endosperm, while the outer daughter cell becomes part of the aleurone layer, with divisions becoming restricted to the anticlinal plane (Walbot, 1994), much as divisions in the protoderm are restricted to the anticlinal plane. Endosperm cell lineage analysis indicates that starchy endosperm and aleurone cells share a common origin throughout development and that the aleurone can *infrequently* divide in the periclinal plane with the internal cell de-differentiating to become starchy endosperm or retaining aleurone identity (Becraft and Asuncion-Crabb, 2000). In *Xcl1* kernels, periclinal divisions produced multiple aleurone layers. It is possible that, like in leaves, the overproduction of XCL1 in aleurones lengthens the time period in which aleurone cells already on a differentiation pathway are competent to divide, thereby producing extra aleurone layers.

Evolutionary implications of *Xcl1*

In nature, countless variations in leaf size, shape, and thickness exist. The *Xcl1* mutation leads to alterations in leaf morphology and anatomy. *Xcl1* leaves are twice as thick but only half as wide as normal leaves. This coordinated change in leaf morphology could be caused by the extra cell layers exerting a biophysical constraint on the *Xcl1* leaf primordium that prevents lamina widening, or by a compensatory mechanism in which extra divisions in *Xcl1* leaves are allocated to the periclinal plane of the protoderm, leaving fewer cell divisions for lamina widening. Spontaneous changes in the *XCL1* gene or pathway could account for differences seen in leaf thickness and width throughout the plant kingdom through alterations in the balance of anticlinal and periclinal divisions. In particular, multiple epidermal layers have evolved in many succulent species as a xeromorphic adaptation (Esau, 1977). In *Peperomia* leaves, the multiple epidermis arises from oblique periclinal divisions in the protoderm (Kaul, 1977), a process very similar to that seen in the *Xcl1* mutant. The daughter cells differentiate according to lineage to form several layers of epidermal cells. Among the grasses, barley and others have multiple aleurone layers. The natural variation required for these evolutionary adaptations may be achieved by altering gradients of cell division and differentiation signals thereby shifting cells into position-dependent differentiation. Our analysis of *Xcl1* indicates that variation in the expression levels of XCL1 plays a key role in the modulation of these signals, allowing for a balance between periclinal and anticlinal divisions and prolongation of the cell division competence in some tissues.

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