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Sectors expressing the homeobox gene *liguleless3* implicate a time-dependent mechanism for cell fate acquisition along the proximal-distal axis of the maize leaf

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SUMMARY

The longitudinal axis of the maize leaf is composed of, in proximal to distal order, sheath, ligule, auricle and blade. The semidominant *Liguleless3-O* (*Lg3-O*) mutation disrupts leaf development at the ligular region of the leaf midrib by transforming blade to sheath. In a previous study, we showed that leaf sectors of Lg3 mutant activity are cell nonautonomous in the transverse dimension and can confer several alternative developmental fates (Fowler, Muehlbauer and Freeling (1996) *Genetics* 143, 489-503). In our present study we identify five Lg3 sector types in the leaf: sheath-like with displaced ligule (sheath-like), sheath-like with ectopic ligule (ectopic ligule), auricle-like, macrohairless blade and wild-type blade. The acquisition of a specific sector fate depends on the timing of Lg3 expression. Early Lg3 expression results in adoption of the sheath-like phenotype at the ligule position (a proximal cell fate), whereas later Lg3 expression at the same position results in one of the more distal cell fates. Furthermore, sheath-like Lg3 sectors exhibit a graded continuum of phenotypes in the transformed blade region from the most proximal (sheath) to the most distal (wild-type blade), suggesting that cell fate acquisition is a gradual process. We propose a model for leaf cell fate acquisition based on a timing mechanism whereby cells of the leaf primordium progress through a maturation schedule of competency stages which eventually specify the cell types along the proximal to distal axis of the leaf. In addition, the lateral borders between Lg3 'on' sectors and wild-type leaf sometimes provide evidence of no spreading of the transformed phenotype. In these cases, competency stages are inherited somatically.

Key words: maize, leaf development, *liguleless3*, maturation schedule, competency, homeobox gene, homeodomain protein

INTRODUCTION

How cell fate is determined is an active area of research in developmental biology. Current research indicates that plant cell fates can be determined by time-dependent or position-dependent mechanisms, or by a combination of the two (reviewed by Sylvester et al., 1996; Freeling, 1992; Dawe and Freeling, 1991). In plants, clonal analysis has been used to study cell lineage and developmental fates of cells in meristems. In general, these studies have shown that although cell lineage does not define cell fate and the developmental fates of cells in the meristem are not fixed (Poethig, 1984; McDaniel and Poethig, 1988; Coe and Neuffer, 1978; Johri and Coe, 1983), developmental fates can be predicted in a general way (Sussex, 1989; Poethig et al., 1986; Scanlon and Freeling, 1997). These analyses and others have suggested that position-dependent mechanisms are important for cell fate acquisition in plants (Poethig, 1989; Dawe and Freeling, 1991; Steeves and Sussex, 1989; van den Berg et al., 1995). However, the relative roles of time- and position-dependent mechanisms for cell fate determination are difficult to address because experimentally uncoupling these mechanisms is troublesome. Maize leaf development has been studied by clonal, genetic and molecular analysis, and thus is a good model for investigating cell fate specification (Poethig, 1987; Freeling, 1992; Sylvester et al., 1996).

Maize leaf development is characterized by three major stages (Sylvester et al., 1990, 1996). In the first stage, a group of approximately 250 founder cells is recruited from the vegetative meristem to form an overlapping ring that will generate the next phytomer (Poethig 1984; Poethig and Szymkowiak, 1995), a repeating segment consisting of the leaf, node, internode and axillary bud (Galinat, 1959). The leaf founder cells make up a subset of the founder cells (Poethig and Szymkowiak, 1995). In the second stage, the leaf founder cells divide at consistent rates to form a leaf primordium that has complete mid- and lateral veins, but is otherwise undifferentiated at the morphological level. Post-primordial growth, consisting of cell division, expansion and differentiation, occurs in the third stage to form the mature leaf. Differentiation occurs

in a graded manner, basipetally from the leaf tip to base, as well as laterally, from the midvein to the two margins.

The two major components of the maize leaf are the proximal sheath and the distal blade (Fig. 1A). The sheath wraps around the culm to provide support to the plant, while the blade acts as the major photosynthetic surface. The blade-sheath boundary is demarcated by the ligular region composed of the ligule and two wedge-shaped auricles. The auricles, in the plane of the leaf, act as a hinge to allow the blade to bend away from the main axis of the plant. The ligule is a flap of epidermal tissue that grows at a right angle to the leaf (Fig. 1A).

The semidominant *Liguleless3-O* (*Lg3-O*; O for original) mutant allele disrupts the leaf at the ligular region of the midrib by transforming blade, auricle and ligule to sheath-like tissue (Fig. 1B; Fowler and Freeling, 1996). The *lg3* gene encodes a homeodomain protein in the *Knotted1*-like (*Knox*) family (Freeling, 1992; Muehlbauer, Fowler and Freeling, unpublished data). Other dominant maize leaf mutants such as *Knotted1-O* (*Kn1-O*) (Freeling and Hake, 1985), *Rough sheath1-O* (*Rs1-O*) (Becraft and Freeling, 1994) and *Liguleless4-O* (*Lg4-O*) (Fowler and Freeling, 1996) exhibit similar blade-to-sheath transformations. Blade, the distal component, exhibits transformation to a more proximal cell type in the transformed region (Freeling, 1992). In *Lg3-O* mutants, cells in the transformed region are sheath-like (proximal) instead of distal ligule, auricle or blade.

The phenotypes of these dominant mutations can be explained by a maturation schedule hypothesis (Freeling, 1992). In short, the maturation schedule hypothesis states that regions of a wild-type leaf pass through a series of competency stages, and the ultimate fate of a cell is determined by the stage when it receives the signal to differentiate. According to this hypothesis, ectopic expression in the leaf of the homeodomain proteins encoded by members of the *knox* gene family, e.g., *kn1* (Smith et al., 1992), *rs1* (Schneeberger et al., 1995) and *lg3* (Muehlbauer, Fowler and Freeling, unpublished results) interfere with acquisition of leaf cell fates. Our model to explain the transformation to more proximal cell identities is that ectopic *knox* gene expression retards the acquisition of competence to express distal cell fates. If the cell fate acquisition process occurs as a chronological progression through the developmental competency stages of sheath, ligule/auricle and blade, then ectopic *knox* gene expression could interfere with this progression and the stage transitions would occur more slowly. In our model to explain the dominant mutant effects, regions of the leaf which ectopicly express the *knox* genes will be in an earlier, or proximal, competency stage when signals to differentiate are received, resulting in differentiation into that more proximal type. Therefore, the maturation schedule hypothesis invokes a continuous, time-dependent mechanism for determining cell fates (Freeling, 1992).

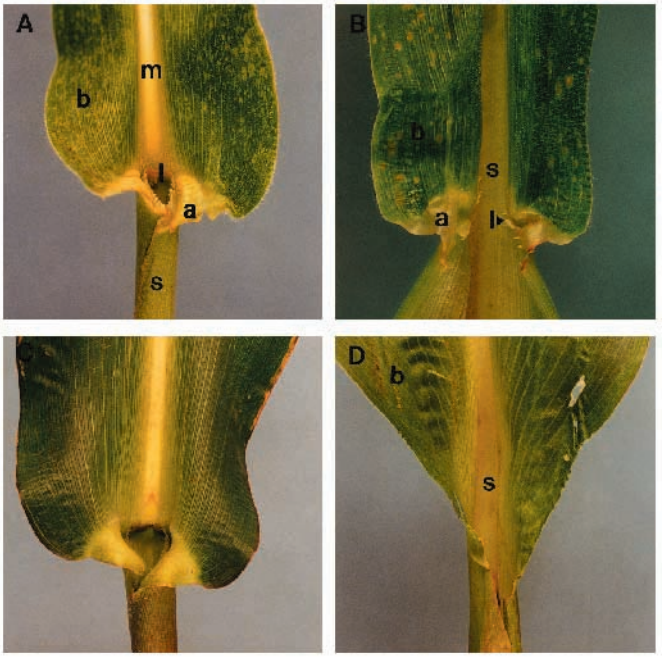


Fig. 1. Wild-type and Lg3 leaves. (A) Wild-type maize leaf. (B) *Lg3-O* leaf. (C) Leaf from a *Mu*-active, *Lg3-Or211* plant. (D) Leaf from a *Mu*-inactive, *Lg3-Or211* plant. a, auricle; b, blade; l, ligule; m, midrib; s, sheath.

In order to dissect the roles of time- and position-dependent regional cell fate acquisition in maize leaves and to test the maturation schedule hypothesis we used our suppressible *Mutator* transposon-based system to coordinately express Lg3 mutant activity and anthocyanin pigmentation in sectors in the leaf (Fowler et al., 1996). In our previous study we showed that Lg3 activity is cell nonautonomous in the transverse dimension. Lg3 activity in the mesophyll alters epidermal cell fates, whereas Lg3 activity exclusively in the epidermis confers no apparent phenotype. In addition, several distinct phenotypic classes of mutant sectors were identified in our previous study, indicating that Lg3 activity can confer more than one cell identity. In this study we show that the timing of sector initiation determines the specific cell fate of the sector, supporting the maturation schedule hypothesis. Thus, we present a model that invokes a role for time-dependent mechanisms for regional cell fate acquisition in the maize leaf.

MATERIALS AND METHODS

Genetic stocks

The semidominant *Lg3-O* mutant allele was obtained from the Maize Genetics Stock Center (Fig. 1B; Perry, 1939; Fowler and Freeling, 1996). The *Lg3-Or211* allele was derived from a *Mutator* (*Mu*) transposable element insertional screen for reversion of the Lg3 phenotype to wild type (Fowler et al., 1996). *Lg3-Or211* is *Mu*-suppressible (Fowler et al., 1996). *Mu*-active plants carrying the *Lg3-Or211* allele exhibit a wild-type phenotype (Fig. 1C). However, in *Mu*-inactive plants, *Lg3-Or211* causes a severe Lg3 phenotype (Fig. 1D). *a1-mum2* is a *Mu*-suppressible allele of the anthocyanin structural gene *a1*. In *Mu*-active plants the *a1-mum2* allele confers a green blade phenotype with a faint red color in the sheath, ligule and midrib region, whereas in *Mu*-inactive plants it confers a deep red/purple coloring throughout the leaf. *Mu* activity was supplied from a line containing only a single copy of the autonomous *Mu* element, [*MuDR-1*(*p1*)] (Chomet et al. 1991; Lisch et al., 1994; Lisch and Freeling, 1995). All alleles of the anthocyanin structural genes necessary to provide anthocyanin pigmentation (*A2*, *Bz1*, *Bz2* and *C2*) were present in the genetic background. In addition, the regulatory alleles *R-ch* and *Pl* were used to cause constitutive expression of the anthocyanin biosynthetic genes in the leaf (Coe et al., 1988).

Generation of anthocyanin-marked Liguleless3 sectors

A maize line containing the *Mu*-suppressible *a1-mum2* and *Lg3-*

Or211 alleles was constructed in a genetic background containing a single copy of *MuDR-1* and the anthocyanin-conditioning alleles *R-ch, Pl, A2, Bz1, Bz2* and *C2* (Fowler et al., 1996). In this background, *Mu*-active plants produce faint red leaves that exhibit a wild-type ligule phenotype. When the single copy *MuDR-1* inactivates itself somatically, sectors of *Mu*-inactivity are produced (Lisch and Freeling, 1995). These *Mu*-inactive sectors exhibit coordinate expression of anthocyanin pigmentation and Lg3 activity (Fig. 2; Fowler et al., 1996).

Analysis of sectored plants

Plants were grown in the fields at Berkeley and San Jose, CA during three consecutive summers. Leaves from *Mu*-active plants which displayed red/purple *Mu*-inactive sectors with a Lg3 phenotype were removed for analysis. Only those sectors that passed through the blade-sheath boundary and exhibited anthocyanin pigmentation in the mesophyll or the mesophyll plus the epidermis were investigated. We showed previously that even large sectors that were restricted to the epidermis exhibited no Lg3 phenotype (Fowler et al., 1996); these sectors were not analyzed in this study. This previous study indicated that Lg3 activity is needed in the mesophyll, but not the epidermis, to cause a mutant phenotype. Sectors were classified as single leaf sectors if they were only observed in a single leaf. Meristematic sectors were scored as sectors observed in more than one leaf (Steffenson, 1968). The inherent nature of anthocyanin marked sectors made it sometimes difficult to observe the sectors in the sheath, node or internode. Therefore, we classified sectors as single leaf if we did not observe a sector in the blade of the next leaf. Sectors were scored at the blade-sheath boundary for their Lg3 phenotype (Fowler et al., 1996), and width and distance from the midrib. Material for scanning electron microscopy (SEM) was prepared according to Sylvester et al. (1990). SEM was conducted at the electron microscopy facility at the University of California, Berkeley.

Scanning electron microscopy analysis of developing ligules

Two week-old seedlings of heterozygous *Lg3-O* and nonmutant siblings in a W23 background were dissected for SEM of the adaxial surface of plastochron 3 through plastochron 9 (P3-P9) leaves. The term plastochron is used to indicate the measure of time it takes the meristem to produce successive leaves. At the time of dissection, plants had 11-12 leaves. The leaf number is counted from the oldest leaf (leaf 1) to the meristem. Therefore, in a plant with 12 leaves a P3 leaf would be labelled P3, L9.

RESULTS

Wild-type and *Lg3-O* ligule development

We compared the timing and rate of ligule development in heterozygous *Lg3-O* mutant and nonmutant siblings by examining the adaxial surface of leaves from plastochrons 3 through 9 (P3-P9) on seedlings containing 11 to 12 leaves. In both heterozygous *Lg3-O* and nonmutant siblings, ligule development was first observed in P7 leaves as a row of periclinal divisions at the blade-sheath boundary, indicating that *Lg3-O* does not alter the timing of ligule initiatian (Fig. 3A,C). We did not observe any anticlinal divisions, indicative of ligule development, in the adaxial surfaces in leaves P3, L9; P4, L8; P5, L7 or P6, L6 from *Lg3-O* or nonmutant siblings (data not shown). Sylvester and colleagues (1990) showed previously that ligule development initiates near the midrib and proceeds in a straight line towards the margin. We observed that *Lg3-O* ligules initiate at a position more distal than the normal ligule position, then curve basipetally towards the normal ligule position (Fig. 3C). In *Lg3-O* plants, the ligule was absent over the midrib region and replaced by sheath-like tissue. Thus, the *Lg3-O* ligule initiates at an incorrect position. SEMs of older P9 leaves showed that the ligule in *Lg3-O* mutant and nonmutant siblings were of approximately the same size, indicating that mutant and nonmutant ligules develop at the same rate (Fig. 3B,D).

Lg3 sector types

Lg3 sectors in wild-type plants were generated and recognized by coordinate *Mu*-suppression of *Lg3-Or211* and *a1-mum2* (Fig. 2; Fowler et al., 1996). We analyzed the anthocyanin-marked Lg3 sectors to determine their phenotypic effects and to correlate their effects with the time at which the sector (and thus Lg3 expression) originated. We identified 26 *Mu*-active plants containing 110 *Mu*-inactive, anthocyanin-marked Lg3 sectors that passed through the blade-sheath boundary region. Sectors were classified based on gross epidermal morphology, as each portion of the maize leaf exhibits distinctive epidermal cell types (Fig. 4; Sylvester et al., 1990; Freeling and Lane, 1994). Five sector types were identified: (1) sheath-like with displaced ligule (sheath-like); (2) sheath-like with ectopic ligule (ectopic ligule); (3) auricle-like; (4) macrohairless blade; and (5) wild type (Fig. 5; Fowler et al., 1996). (1) Sheath-like sectors developed sheath-like cells in the position normally occupied by ligule, auricle and blade. A new blade-sheath boundary alongside the sector was present and often a displaced ligule grew at the boundary (Fig. 5A). SEMs of the sheath-like sectors showed noncrenulated rectangular sheath-like cells within the sector bordered by a displaced ligule outside the sector (Fig. 5B). (2) Ectopic ligule sectors exhibited blade and auricle transformed to sheath-like tissue with ligule fringes either in the blade or auricle (Fig. 5C,D). However, a normal ligule developed in the proper position in these sectors.

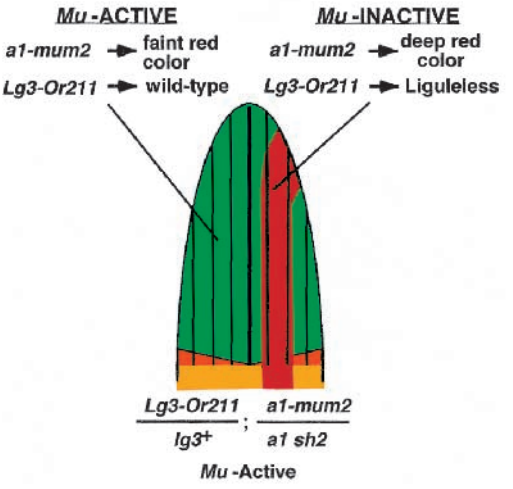


Fig. 2. Schematic of a leaf from a *Mu*-active, *Lg3-Or211, a1-mum2* plant. In these plants, a single copy of *MuDR-1* provides the *Mutator* activity, and the *Lg3-Or211* allele causes a wild-type phenotype, and the *a1-mum2* allele produces a faint red phenotype. When *MuDR-1* inactivates itself somatically, *Mu*-inactive sectors of *Lg3-Or211* cause a Lg3 phenotype (not shown in the figure) and *a1-mum2* causes the production of anthocyanin. The blade is represented by green, the auricle/ligule by orange, the sheath by yellow and the *Mu*-inactive sector by red. This figure is reproduced from *Genetics* **143,** 489-503 (1996).

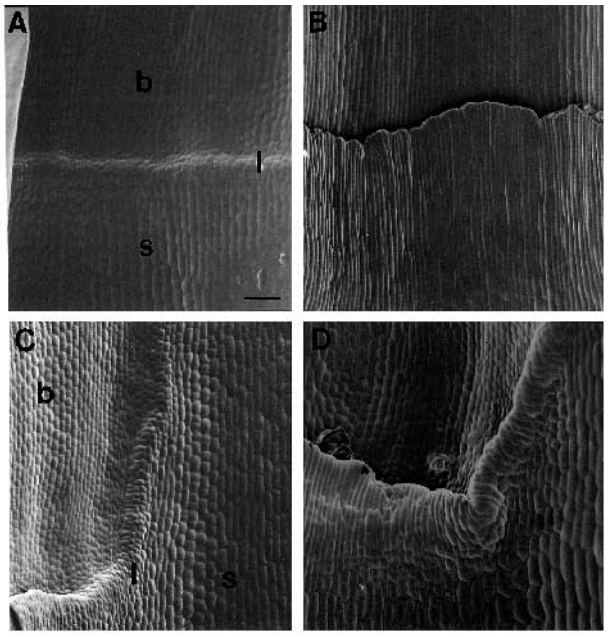


Fig. 3. Scanning electron micrographs of wild-type and *Lg3-O* ligule development. (A) Wild-type leaf (P7, L5) showing a small ridge of cells that will develop into the ligule. (B) Wild-type leaf (P9, L3) showing a more developed ligule. (C) *Lg3-O* leaf (P7, L5) showing a small ridge of cells that will develop into a ligule. (D) *Lg3-O* leaf (P9, L3) showing a more developed ligule. b, blade; l, ligule; s, sheath. Scale bar, A, 112 μm; B, 108 μm; C, 108 μm; D, 105 μm.

(3) Auricle-like sectors exhibited squarish, hairy auricle-like cells extending into the blade. The ligule and auricle were wild-type in these sectors (Fig. 5E,F). (4) Macrohairless blade sectors, not previously described, exhibited normal ligule and auricle development, however, the blade within the sector had no macrohairs (Fig. 5G,H). Macrohairs are normally positioned over rows of bulliform cells and grow out of approximately 10-20 basal cells (Fig. 4A). (5) Wild-type sectors exhibited no mutant phenotype (Fig. 5I,J). These data indicate that expression of the Lg3 mutant activity can confer multiple, distinct cell fates.

Sectors originating in single leaves and meristems

In order to determine whether the timing of sector initiation correlated with specific sector phenotypes we took advantage of the fact that the size of the sector is related to the time in development that the sector arose; smaller sectors are initiated later than larger sectors (Poethig, 1984). We classified sectors into two classes: those that initiated in a single leaf, and those that arose in the meristem and thus resulted in sectors appearing in multiple leaves (Steffenson, 1968). All five sector types were observed in the single leaf class. We therefore subdivided the class into sector type, and asked if the sector width correlated with specific cell or regional fate (Table 1). In the single leaf sector category, 37 sheath-like, 13 ectopic ligule, six auricle-like, 18 macrohairless blade and 19 wild-type sectors were observed. Of the single leaf sectors, the sheath-like sectors exhibited the largest average width at about 8% of a half leaf (Table 1). Ectopic ligule, auricle-like and macrohairless blade were similar in average width at about 4% of a half leaf. Wild-type sectors were the smallest, exhibiting an average width of 2.6% of a half leaf. The lateral positions of the specific sector types in relation to the midrib did not exhibit clustering, indicating that the ability to express one of the five transformation phenotypes is not due to its lateral position in the leaf (Table 1). These data indicate that there is a general trend relating the timing of sector initiation to the transformation phenotype present in the sector. Large sectors (early Lg3 expression) are correlated with a sheath-like cell fate, whereas medium-sized sectors (later Lg3 expression) are correlated with ectopic ligule, auricle-like, macrohairless blade sector types, and the smallest sectors (latest Lg3 expression) are correlated with the wild-type sector type.

Table 1. Single leaf sectors

Sector type	*n*	% average width±s.d.	% distance from midrib (range)
Sheath-like	37	7.8±0.9	0 - 88
Ectopic ligule	13	4.4±0.8	14 - 64
Auricle-like	6	4.0±0.6	25 - 70
Macrohairless blade	18	3.6±0.4	28 - 68
Wild-type	19	2.6±0.3	22 - 93

Meristematic sectors initiate in the meristem proper and pass through more than one phytomer (Steffenson, 1968). Therefore, meristematic sectors represent the earliest sectors in leaf organ growth. We identified 25 meristematic sectors that passed through 115 leaves (Table 2). Of these 115 leaves, 110 exhibited sheath-like with displaced ligule (sheath-like) sectors, and five contained sheath-like with ectopic ligule (ectopic ligule) sectors (Table 2). Sheath-like, meristematic sectors were the largest type of sector exhibiting an average width of 12% (range 4-42%) of a half leaf (Table 2). The five meristematic ectopic ligule sectors were also relatively large with an average width of 8% (range 5-11%) of a half leaf. Because meristematic sectors are the earliest possible sectors examined here, these data also indicate that early sectors result in the sheath-like phenotype.

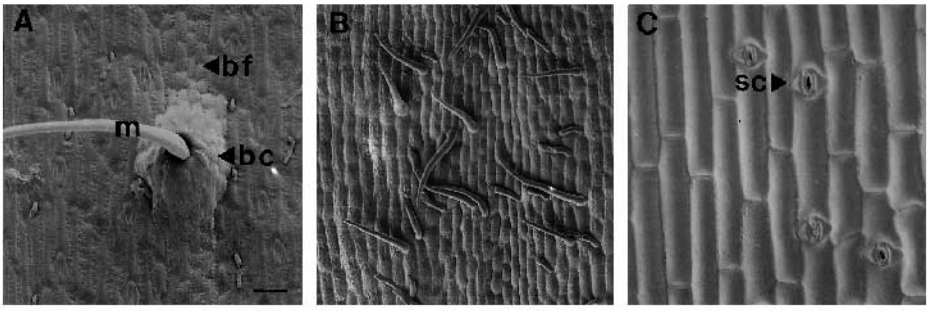


Fig. 4. Wild-type leaf tissue types. (A) SEM of leaf blade tissue from a wild-type plant. (B) SEM of auricle tissue from a wild-type plant. (C) SEM of sheath tissue from a wild-type plant. bf, bulliform cells; bc, basal cells; m, macrohair; sc, stomatal complex. Scale bar, A, 116 μm; B, 123 μm; C, 125 μm.

Table 2. Meristematic sectors

Sector type	No. of sectors	No. of leaves*	% average width±s.d.
Meristematic	25	115	±
Sheath-like		110	12.2±0.9
Ectopic ligule		5	7.8±1.03

*Number of leaves that the sectors pass through.

Continuum of phenotypes within sheath-like sectors

SEMs of the epidermal surface of sheath-like sectors revealed a continuum of phenotypes along the longitudinal dimension as the sector progressed from the sheath proper to the tip of the leaf (Fig. 6A-G). Cells in the the sheath proper were undisturbed by ectopic Lg3 expression (Fig. 6G). At the blade-sheath boundary, cells within the sector also exhibited sheath-like characteristics with displaced ligule-like tissue appearing alongside the sector (Fig. 6F). Further examination of the sheath-like sectors revealed, in order along the P-D axis, the following phenotypes: sheath-like cells without displaced ligule (Fig. 6E); mixture of blade- and sheath-like cells (Fig. 6D); macrohairless blade (Fig. 6C); and wild-type blade cells (Fig. 6B). The order of these cell types was consistent among sectors. Similar distributions of mutant phenotypes, although initiating at a different point along the continuum, were also observed in the other sector types. For example, auricle sectors exhibited auricle phenotypes, which then were gradually replaced by more distal phenotypes toward the distal tip of the sector (data not shown). These data indicate the variable

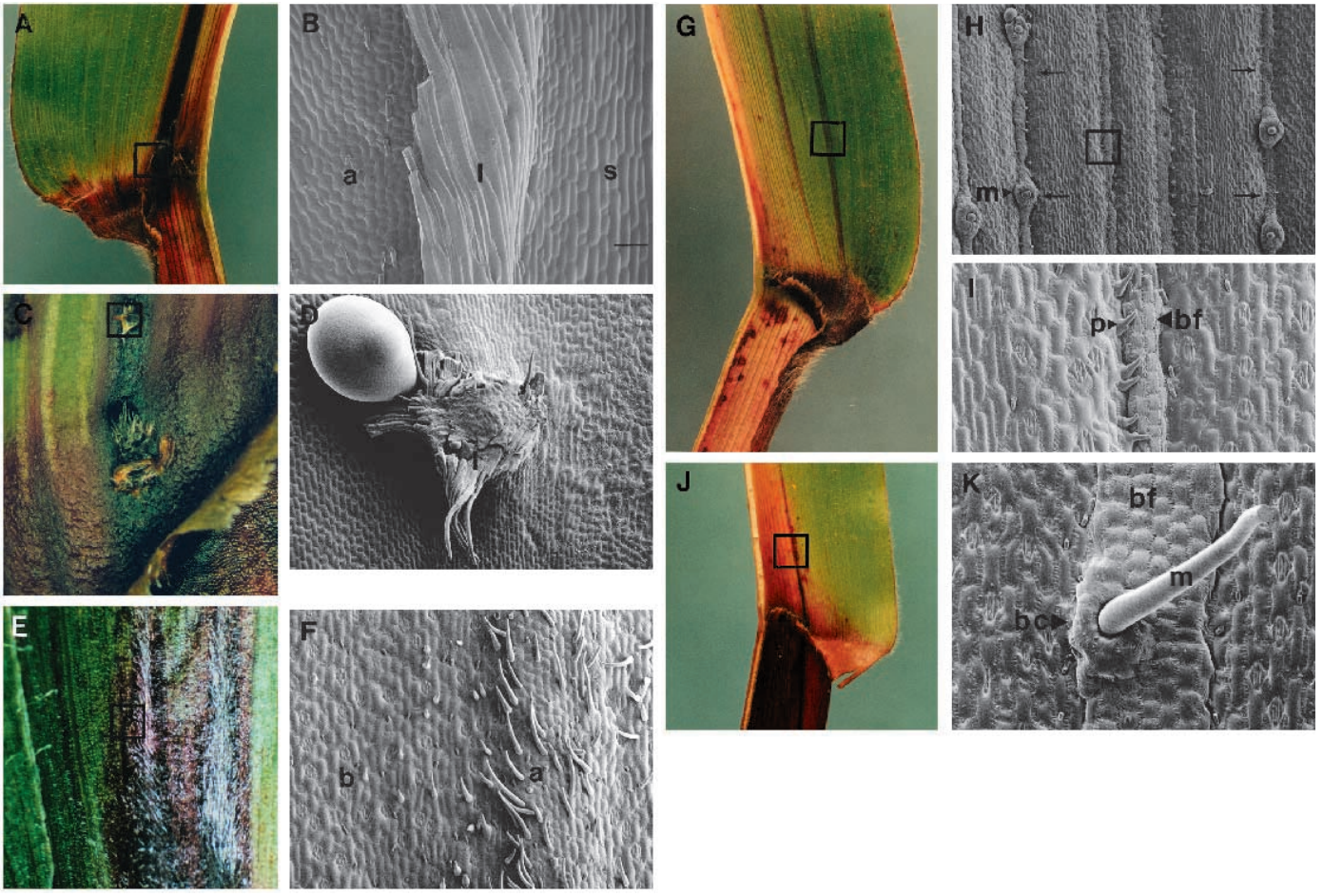
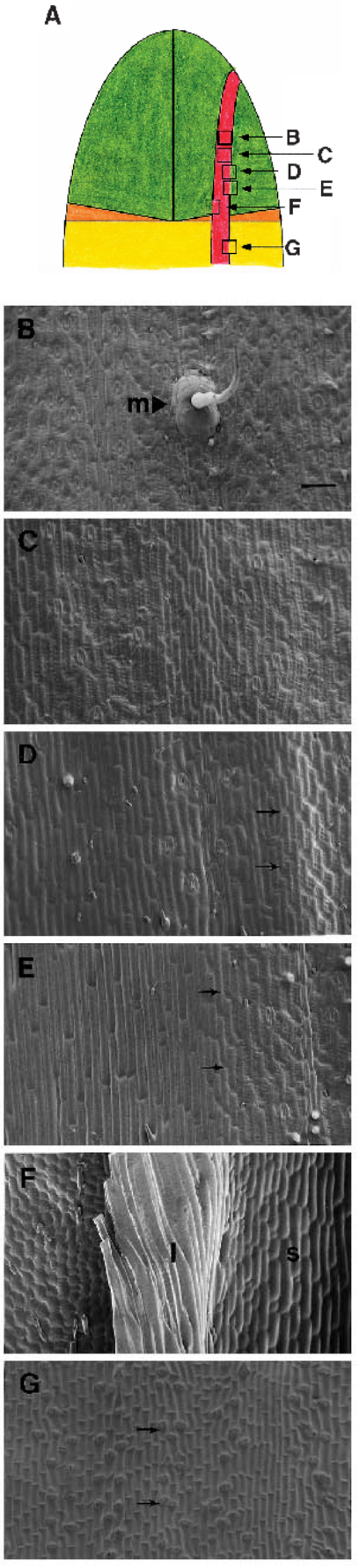


Fig. 5. Examples of the five Lg3 mutant phenotypes expressed in anthocyanin-marked sectors. The boxed areas in A,C,E,G,H and J are shown enlarged in B,D,F,H,I, and K respectively. (A) Sheath-like sector showing disruption of the ligule at the blade-sheath boundary. (B) SEM of the boundary of a sheath-like sector showing the uncrenulated, rectangular sheath-like cells within the sector bordered by ligule tissue. Outside the sector auricle cells are present. (C) Blade with an ectopic ligule sector showing two prominent patches of ectopic ligule. (D) SEM of the ectopic ligule with blade cells on both the right and left sides of the ectopic ligule. The large bubble is an artifact of sample preparation. (E) Auricle-like sector showing hairy auricle-like cells in the blade. (F) SEM of the auricle-like sector shows hairy auricle-like cells bordered by blade cells. (G) Macrohairless blade sector showing no disruption of ligule or auricle. (H) SEM of macrohairless blade sector showing that macrohairs are not present within the sector. The anthocyanin-marked sector covers the region between the macrohairs, as indicated by the arrows. The macrohairs in this SEM were broken off during the sample preparation process. (I) SEM of macrohairless blade sector showing that the blade cells within the sector are normal. Note that the bulliform cells exhibit a normal appearance except for the absence of basal cells and macrohairs. (J) Wild-type blade sector showing normal ligule, auricle and blade. (K) SEM of a wild-type blade sector showing normal crenulated, rectangular blade cells and a macrohair. a, auricle; b, blade; bf, buliform cells; l, ligule; m, macrohair; s, sheath. Scale bar, B, 99.4 μm; D, 348 μm; F, 238 μm; H, 553 μm; I, 137 μm; K, 125 μm.



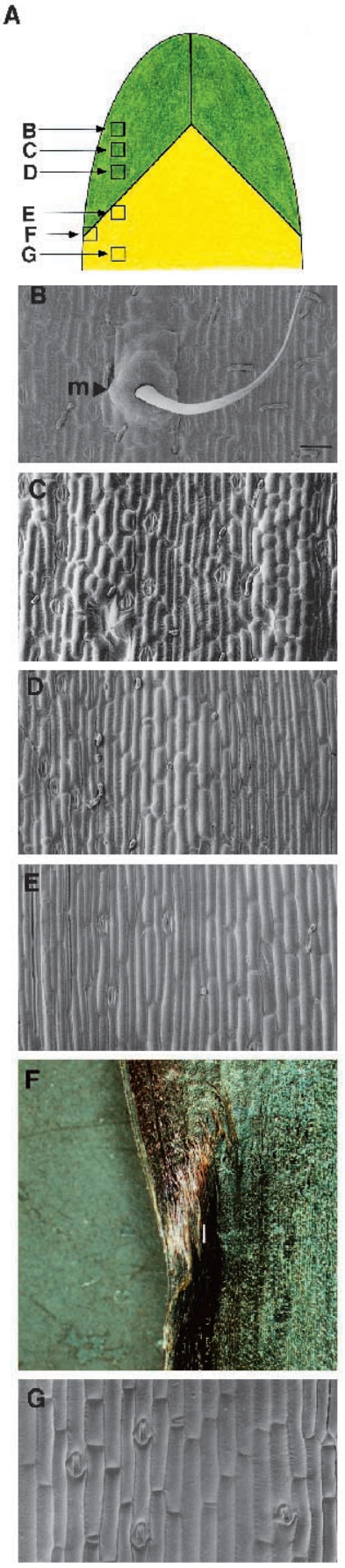
phenotype effect of Lg3 is consistently distributed in an orderly gradient in sectors expressing Lg3 activity.

We also observed a lateral distribution of variable phenotypes in the sheath-like sectors from the inside of the sector to the sector border. The sheath-like phenotype appears to be a distinct, single cell type in the lateral center of the sector and a mixture of cell types closer to the sector border. For example, at the sector border of sheath-like sectors there were small squarish auricle-like cells mixed with rectangular larger sheath-like cells and displaced ligule, whereas in the middle of the sector the cell type is predominantly sheath-like (Fig. 6F). In addition, as the sheath-like sector progressed toward the tip of the leaf the cells at the sector border became a mix of sheath- and blade-like cells, whereas the cells in the middle of the sector remained more sheath-like (Fig. 6D). The other sector types did not show this continuum of phenotypes from the middle of the sector to the border. These observations indicate that the effect of ectopic Lg3 expression can be altered by proximity to wild-type cells/tissue.

Continuum of phenotypes in the the transformed region of *Mu*-inactive *Lg3-Or211* leaves

Mu-inactive plants carrying the *Lg3-Or211* allele exhibit a severe Lg3 phenotype (Fig. 1D). To determine whether the distribution of mutant developmental fates along the P-D axis of the Lg3 sectors was also a feature of these plants, we examined leaves of *Mu*-inactive plants carrying the *Lg3-Or211* allele (Fig. 7). This revealed a continuum of phenotypes very similar to that observed in the sheath-like sectors. The sheath proper appeared to be undisturbed by the expression of the Lg3 phenotype; i.e., long rectangular, uncrenulated epidermal sheath cells were observed (Fig. 7G). At the new blade-sheath boundary a small remnant of ligule was present but only at the leaf margins (Fig. 7F). As the transformed region of sheath-like cells entered the blade, the displaced ligule is absent (Fig. 7E). Close examinination of this region revealed rectangular, sheath-like cells. Continuing along the P-D axis the following phenotypes were observed: mixture of blade and sheath cells (Fig. 7D), macrohairless blade (Fig. 7C), and wild-type blade (Fig. 7B). As in the sectors, a lateral distribution of cell types was observed. A single sheath-like cell type was present in the transformed region at the midrib (middle) and a mixture of cell types were observed towards the lateral edges of the transformed region (data not shown).

Fig. 6. Phenotypes within a sheath-like sector. (A) Schematic of a leaf blade with a sheath-like sector indicated in red. Boxes indicate the positions that the SEMs in B-G are from. (B) Wild-type blade tissue in the center of the sector. (C) Macrohairless blade tissue in the center of the sector. (D) A mixture of sheath and blade tissue occurs within the sector, and outside the sector boundary normal blade cells are observed. Arrows indicate right border of the sector. (E) Sheath-like tissue occurs within the sector whereas outside the sector boundary normal blade tissue is observed. Arrows indicate right border of the sector. (F) Sheath-like tissue occurs within the sector, with a displaced ligule at the sector border. Outside the sector auricle tissue is observed. (G) Sheath tissue within the sector is identical to sheath tissue outside the sector. The arrows indicate the right border of the sector. l, ligule; m, macrohair; s, sheath. Sheath tissue is represented by yellow, auricle/ligule tissue by orange, and blade tissue by green. Scale bar, B, 114 μm; C, 108 μm; D, 102 μm; E, 104 μm; F, 99.4 μm; G, 298 μm.



DISCUSSION

Lg3 mutant activity confers no specific cell type or leaf regional identity information

We generated sectors of Lg3 mutant activity to determine the number of cell types the ectopic activity of Lg3 can confer. Our data indicate that Lg3 expression can confer multiple cell fates or regional identities in the leaf. We observed five sector types in this study: (1) sheath-like with displaced ligule (sheath-like), (2) sheath-like with ectopic ligule (ectopic ligule-like), (3) auricle-like, (4) macrohairless blade and (5) wild type (Fig. 5). If cell types are graded, sheath being proximal, and ligule, auricle and blade being distal, our data indicate that Lg3 expression shifts cell fate decisions in the proximal direction. Also, these data indicate that Lg3 expression can confer more than one cell fate.

The timing of Lg3 sector initiation and not sector position, determines the cell or regional identity

We determined that the timing of sector initiation correlated with the ultimate cell fate within the sector. Single leaf sectors represent clonal events which occurred within cells of the developing leaf primordia. Sectors initiating early in the development of the leaf are large compared to sectors initiating later in development, as a greater number of cell divisions occurs in the early clones. Therefore, sector sizes in the single leaf sectors allow an estimate of the relative timing of sector initiation. Our data on the single leaf sector category show that large sectors, corresponding to early Lg3 expression in leaf development, give rise to sheath-like sectors, and smaller sectors corresponding to later Lg3 expression, give rise to either ectopic ligule, auricle, or macrohairless blade sectors. Finally, the smallest sectors (latest Lg3 expression) result in wild-type blade sectors. These data reveal a clear trend: sectors intitiated early in leaf development give rise to the most proximal cell fates, whereas sectors initiated later in development result in one of the distal cell fates. Further evidence to support this phenotypic dependence on timing of Lg3 expression can be seen in the meristematic sector class. Meristematic sectors represent a clonal event causing Lg3 expression before the leaf was initiated. Thus, these phenotypic sector types were associated with Lg3 expression at the earliest possible time in leaf development. We observed 25 meristematic sectors spread through 115 leaves. Of these leaves, 110 exhibited sheath-like sectors and five leaves exhibited ectopic ligule sectors (Table 2). Auricle-like, macrohairless blade and wild-type sectors were not seen in the meristematic sector class, indicating that Lg3 expression before leaf

Fig. 7. Phenotypes of a leaf from a *Mu*-inactive, *Lg3-Or211*, *a1-mum2* plant. (A) Boxes indicate regions in a leaf from a *Mu*-inactive, *Lg3-Or211* plant examined by SEM and shown in B-G. (B) Wild-type blade tissue is observed distal to the transformed region. (C) A macrohairless blade phenotype is observed distal to the region of blade-to-sheath transformation. (D) A mixture of blade and sheath tissue is observed near the boundary of the tranformation. (E) Sheath-like tissue is observed within the region of blade-to-sheath transformation. (F) A remnant of ligule is observed on the margin at the position where a normal ligule is positioned. (G) Sheath tissue is observed in the sheath proper. m, macrohair; l, ligule. Sheath tissue and the blade to sheath transformation are represented by yellow, and blade tissue by green. Scale bar, B, 103 μm; C, 111 μm; D, 112 μm; E, 116 μm; G, 118 μm.

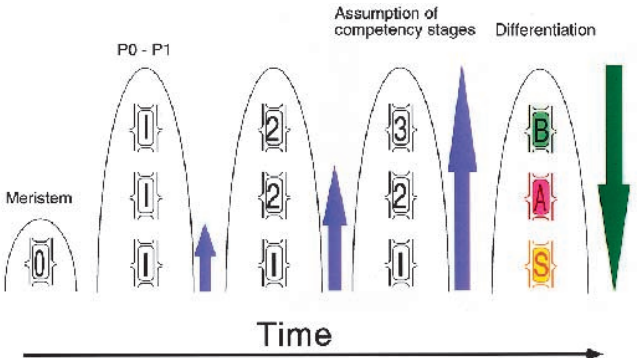


Fig. 8. The maturation schedule hypothesis. Within the meristem, the cells are in the most proximal competency stage signified by the number 0. As the founder cells are initialized and grow into a leaf primordium (P1) the cells are at a competency stage of 1, the sheath competency stage. Subsequently, the cells that will be blade and ligule/auricle progress to a competency stage of 2, the ligule/auricle stage, while the cells that will be sheath remain in the sheath competency stage. Still later, the cells that will be blade acquire the competency stage of 3, the blade competency stage. At this time, all the cells in a particular region of the primordium will have attained their proper competency stages and differentiation proceeds basipetally, with cells adopting fates according to their current competency stage. S, sheath; A, ligule/auricle; B, blade.

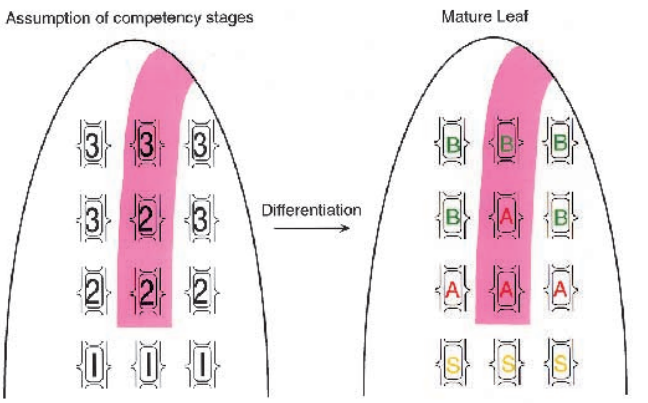


Fig. 9. An auricle sector explained by the maturation schedule hypothesis. Auricle sectors are characterized by a blade-to-auricle transformation. Expression of Lg3 in the sector, indicated by red, has slowed down the acquisition of competency stages within the sector. The expression of Lg3 inhibits the ability of cells within the sector to progress to the blade competency stage. Outside the sector the acquisition of competency stages occurs at a normal rate. Upon differentiation, the cells within the sector differentiate into more proximal cell types (e.g., auricle) than cells outside the sector. 1, sheath competency stage; 2, ligule/auricle competency stage; 3, blade competency stage; S, sheath; A, ligule/auricle; B, blade.

initiation results only in sectors with sheath-like character. In addition, sheath-like sectors exhibit a similar phenotype to that of leaves from *Mu*-inactive, *Lg3-Or211* plants, in which Lg3 expression is always 'on', and thus corresponds to the early, large sector types. Our data indicate that early continuous Lg3 expression in leaf primordia results in a sheath-like phenotype. Taken together, these data indicate that the sheath-like sectors initiate earlier in development than the ectopic ligule, auricle-like and macrohairless blade sector types and that the wild-type sectors arise even later in development.

The lateral positions (relative to the leaf midrib) of the sectors at the blade-sheath boundary were not correlated with specific sector phenotypes. Our data show that the five sector types can be found at most positions in the lateral dimension of the leaf (Table 1). For example, the largest sheath-like sectors and smallest wild-type sectors can be found in the same lateral position (data not shown). Therefore, the lateral position of Lg3 expression does not govern the phenotype of the transformed region of the leaf. Rather, our data indicate that identical positions in the leaf are transformed to different phenotypes dependent on the size of the Lg3 sector, likely due to the time at which Lg3 expression is initiated.

Ectopic expression of *lg3* and other *kn1*-like genes result in distal-to-proximal transformation phenotypes

The SEMs of developing ligules from *Lg3-O* mutant plants show that the ligule, auricle and blade are transformed to a proximal cell type of sheath-like cells, indicating that Lg3 mutant activity alters normal cell fate decisions. The timing and rate of ligule development in *Lg3-O* and nonmutant siblings is similar, indicating that the Lg3 phenotype is due to the establishment of the sheath and ligule cell types at the wrong positions on the leaf. The *lg3* gene is a member of the *knox* family of maize homeobox genes, and dominant *Lg3* alleles cause ectopic expression of the *lg3* gene in the leaf (Muehlbauer, Fowler and Freeling, unpublished results), providing a likely basis for the Lg3 activity and phenotypes.

Our data support the general trend revealed by studies on other homeobox genes. Several other maize dominant neomorphic mutations in the maize *knox* genes (i.e., *Kn1-O*, *Rs1-O* and *Lg4-O*) transform distal cell types to more proximal cell types (Becraft and Freeling, 1994; Fowler and Freeling 1996; Freeling, 1992). Furthermore, transgenic tobacco plants expressing *kn1* create meristematic regions on the leaf blade (Sinha et al., 1993), and transgenic *Arabidopsis* overexpressing an *Arabidopsis kn1*-like gene, *knat1*, exhibit ectopic meristems on the leaves (Chuck et al., 1996). Also, ectopic expression of the *kn1* orthologue from barley, the dominant *hooded* mutation, causes floral meristems on the awn (Muller et al., 1995). In addition, the Arabidopsis *shoot meristemless* gene is necessary for vegetative meristem maintenance, and is a member of the *knox* gene family in *Arabidopsis* (Barton and Poethig, 1993; Clark et al., 1996; Long et al., 1996; Endrizzi et al., 1996). Taken together, these data indicate that ectopic expression of the *knox*-like genes, and their corresponding homeodomain proteins, transforms tissue into a more proximal cell type, with the meristem being the ultimate proximal cell group.

Leaf development and the maturation schedule hypothesis

The maturation schedule hypothesis states that leaf founder cells initiate in the most proximal stage (sheath) and that they progress through a series of competency stages during leaf development from sheath to ligule/auricle to blade before the leaf differentiates (Freeling, 1992). The concept of competency is the major focus of this hypothesis, referring to cells that have not differentiated but have the ability to respond to differentiation signals. We describe the model in greater detail here (Fig. 8). Meristematic cells begin at a competency stage distinct

from, and temporally prior to the sheath competency stage. In the founder cells or P1 (primordial) leaves, cells assume or are in the early (proximal) competency stage of sheath. Thus, all cells in the primordium have the potential to express the sheath fate because all cells go through a sheath competency stage. Over the course of primordial leaf development (stage two), the distal cells that will be ligule/auricle and blade progress to the ligule/auricle competency stage, whereas cells that will become sheath remain in the sheath competency stage. Still later in time, only cells that will be blade progress farther to the blade competency stage; cells that will be ligule/auricle remain in the ligule/auricle competency stage. Thus, by the end of stage two, the blade-ligule/auricle-sheath pattern is established. Finally, differentiation occurs bidirectionally, basipetally from leaf tip to base and laterally from the midrib of the leaf to the margin. Therefore, cells that will become blade go through the sheath, ligule/auricle and into blade competency stages before they are signalled to differentiate. Founder cells initiate first at the midrib, whereas more proximal and marginal regions initiate later (Poethig, 1984; Steffenson, 1968; Sylvester et al., 1990). Our model suggests that the founder cells that are initialized first (i.e., midrib) will enter the maturation schedule first. Therefore, these cells will reach the blade competency stage before more proximal and marginal founder cells.

We can explain the phenotypic variation in the Lg3 sectors in terms of this maturation schedule model. Our data show that when Lg3 is expressed in the meristem or leaf founder cells (meristematic sectors or large single leaf sectors), sheath-like sectors result. Our interpretation of these results is that ectopic Lg3 expression inhibits the normal progression of cells through subsequent competency stages; thus, the Lg3-expressing founder cells, which are all at the sheath stage, do not progress correctly to later stages, and eventually adopt a sheath-like fate. These data also indicate that sheath is the initial competency stage, because the earliest sectors (meristematic) were invariably sheath like. However, smaller sectors, in which Lg3 expression initiates later in time are in a later competency stage when normal continuation of the competency schedule is inhibited by Lg3 expression. Cells in these sectors have already begun the wild-type process of progression from the sheath competency stage to the ligule, auricle or blade competency stages when Lg3 inhibition originated. Thus, the cells in these sectors eventually adopt the more distal ectopic ligule, auricle-like, macrohairless blade and wild-type sector phenotypes. Our model implies that the specific fate ultimately exhibited in the Lg3 sector is based on the timing of sector initiation, due to inhibition of the competency progression beginning at different points in the maturation schedule. For example, Fig. 9 illustrates an auricle sector in terms of the maturation schedule hypothesis. Lg3 expression always causes adoption of more proximal cell fates because distal cell fates are the more advanced stages of the progression that is inhibited by Lg3 expression. It must be emphasized that inhibition of the schedule progression by Lg3 does not slow down differentiation and morphogenesis in the leaf; our data show that *Lg3-O* and wild-type ligules initiate and develop at similar times and rates. Rather, Lg3 expression inhibits a regulated progression of competency over time (i.e., the maturation schedule), and the extent of the progression through these events is a primary determinant of cell fate in the leaf. Therefore, our results implicate a role for a timing mechanism for determining cell fates in leaf development.

Examples of this type of time-based mechanism for development have been previously observed in animals and plants. Muscle formation in *Xenopus laevis* embryos can be explained by a similar maturation schedule. The studies on *Xenopus* show that the embryo follows a schedule of events based on a timing mechanism (Cooke and Smith, 1990). Degradation of proteins or mRNA has been proposed as the molecular basis for such a timing mechanism (Gurdon, 1992). In *Arabidopsis*, trichome production on the abaxial side of the leaf has been shown to be controlled by the age and not the size of the plant (Telfer et al., 1997)

Previous characterization of the maize *Hairy-sheath-frayed1-O* (*Hsf1-O*) mutation led to the initial suggestion that a timing mechanism was operative in controlling fates during development (Bertrand-Garcia and Freeling, 1991; Freeling et al. 1992). *Hsf1-O* is a heterochronic mutation that prolongs the juvenile phase of development in the maize shoot. Furthermore, it also causes appearance of sheath-like prongs in blade margins. The authors proposed that the sheath is a 'juvenile' blade and that *Hsf1-O* prolonged the 'juvenile' stage of blade development, resulting in a transformation of blade to sheath at the margins. Our data provide an independent and more rigorous test to support this initial argument for the importance of time in determining cell fate in leaf development. Further, our maturation schedule hypothesis extends and adds details to the initial model.

LG3-induced leaf transformations occur in a continuous array of phenotypes from sheath to blade

Sheath-like sectors and leaves from *Mu*-inactive *Lg3-Or211* plants reveal a similar continuous array of phenotypes from the sheath proper to the distal blade (Fig. s 6 and 7). From the base of the sheath to the tip of the blade these phenotypes are observed in the following order: sheath, sheath with ligule, sheath, sheath with a mixture of blade, macrohairless blade and wild-type blade. This type of continuum of phenotypes can be explained by our model if Lg3 ectopic expression slows but does not halt the acquisition of competency stages. Leaf differentiation occurs in a basipetal fashion (tip to base), whereas our model suggests that the establishment of competency stages proceeds in an acropetal fashion (base to tip). Founder cells at the midrib initiate before more proximal and marginal founder cells (Poethig, 1984; Steffenson, 1968; Sylvester et al., 1990), such that there is an age continuum (i.e., time since founder cell initialization) within the founder cells from tip to base and midrib to margin. Lg3 expression has less of an effect on the phenotype at the blade tip than proximal or marginal leaf positions because the founder cells that will become the distal blade cells will have more time, relative to the time proximal and marginal founder cells have, to go through the competency stages before differentiation. According to our hypothesis, cells at the blade tip have a wild-type appearance because they had already advanced to the blade competency stage when Lg3 expression had its effect, whereas cells in the proximal portion of the blade were 'hung-up' in a more proximal competency stage and are not able to differentiate into blade. Therefore, Lg3 ectopic expression in *Mu*-inactive, *Lg3-Or211* plants and sheath-like sectors results in a continuum of phenotypes from leaf base to tip and not just a single cell type. Interestingly, the 'later' sector types; ectopic ligule, auricle-like, macrohairless blade and wild type appear to be subsets of the entire continuum of phenotypes seen in

leaves from *Mu*-inactive, *Lg3-Or211* plants. Overall, our data indicate that the Lg3 sector phenotypes are a continuum, not a specific transformed cell fate.

A note on the cell-autonomy/nonautonomy of cell fate in the leaf

It is exceedingly difficult to know whether or not cells within our Lg3-transformed sectors signal their cell fates to one another. Judging from the literature that implicates position within a signalling field as important for cell fates (Dawe and Freeling, 1991) one might assume nonautonomy within our sectors. However, in some of our sectors, the borders between our transformed leaf tissue and adjacent 'normal' tissue are tight or reasonably tight lines of phenotype (Fig. 5; Fowler et al., 1996). Our interpretation is that the transformation occurred as a result of the maturation schedule, (i.e., competency infomation), respecting or largely respecting the sector boundary lines between Lg3 cells and wild-type cells. These results indicate that competency stages can be inherited somatically.

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