Interactions of *liguleless1* and *liguleless2* Function During Ligule Induction in Maize

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Manuscript received April 25, 1996
Accepted for publication September 9, 1996

ABSTRACT

The maize ligule is an adaxial membranous structure on the leaf that develops at the boundary of the sheath and blade. The ligule and the associated auricle are dispensable structures, amenable to genetic manipulation. We present here a genetic analysis of *liguleless1* (*lg1*) and *liguleless2* (*lg2*), the two genes known to be uniquely necessary for ligule and auricle development. We show that both reference mutant alleles, *lg1-R* and *lg2-R*, are null alleles. The double mutant phenotype suggests that *lg1* and *lg2* act in the same pathway. Indeed, the dosage of a functional allele at either gene affects the null phenotype of the other. While *lg1* function has previously been shown to be cell-autonomous, here we show that the *lg2-R* phenotype is cell-nonautonomous, suggesting *lg1* and *lg2* play different roles in the ligule-auricle induction mechanism. We present a model in which early *lg2* function specifies the precise position where ligule and auricle will develop. Later *lg2* function interacts with *lg1* function (either directly or indirectly) to transmit and receive a make-ligule-make-auricle inductive signal.

THE leaf of maize (*Zea mays* L.) is typical of the grasses. It is separated into blade and sheath by the ligule-auricle region (Figure 1). The ligule is a membranous fringe of epidermal tissue that grows perpendicular out of the plane of the leaf. The auricle encompasses all tissue layers and acts as a hinge that allows the leaf blade to bend out from the main axis.

Our current understanding of maize leaf development involves three general phases (SYLVESTER *et al.* 1990; FREELING 1992; SMITH and HAKE 1992; POETHIG and SZYMKOWIAK 1995; HARPER and FREELING 1996). First, a group of cells in the meristem is recruited to become the next phytomer, the repeating unit of leaf, node, internode and axillary bud. The subset of these cells that will become the leaf are termed the "leaf founder cells" [defined in POETHIG (1984)]. Second, the founder cells all divide about equally to establish the leaf primordium, which can be seen as a bulge on the meristem flank. Third, a basipetal polar differentiation begins accompanied by a lateral polar differentiation from the middle toward the margin.

The earliest identifiable morphological features on a maize leaf primordium are three regions of different adaxial epidermal cell shapes (SYLVESTER *et al.* 1990). These correspond to the three regions on the mature leaf: the blade, the ligular region, and the sheath. The middle region, where the ligule and auricle eventually differentiate, represents an important marker on the leaf that we call the blade-sheath boundary. At an early point in development, this boundary occupies a disproportionately large region of the primordium (SYLVESTER *et al.* 1990). Because it appears so early, and because no mutants in maize remove it, we argue that establishment of the blade-sheath boundary is a fundamental defining feature of a maize leaf, and perhaps of all grass leaves (FREELING 1992). The dispensable ligule and auricle are elaborated later than the establishment of the blade-sheath boundary; however, they may utilize aspects of this boundary as cues for their development.

Ligule differentiation begins when a leaf primordium is in its fourth to sixth plastochron, and differentiation is occurring at the tip of the blade. First, anticlinal divisions (new wall inserted perpendicular to the plane of the leaf) appear on the adaxial surface of the leaf to form a band of small cells termed the preligular band (SHARMAN 1941, 1942; HAKE *et al.* 1985; BECRAFT *et al.* 1990; SYLVESTER *et al.* 1990; BECRAFT and FREELING 1991). Formation of the preligular band is directional beginning at two foci, one on either side of the midrib, and proceeding outward toward the margins and inward over the midrib (SYLVESTER *et al.* 1990). Following preligular band formation are the periclinal divisions (new walls inserted parallel to the plane of the leaf) that allow the ligule to grow out of the plane of the leaf (SHARMAN 1941, 1942; BECRAFT *et al.* 1990). These periclinal divisions are also laterally directional, again following the general context of leaf differentiation from midrib to margin (HAKE *et al.* 1985). These divisions result in the final differentiation of the ligule. The auricle is elaborated from a subset of internal cells in the preligular band that forms between the blade and the ligule. The periclinal divisions and the elaboration of the auricle occur at a time when the leaf blade is completing differentiation, and differentiation of the leaf sheath is just beginning.

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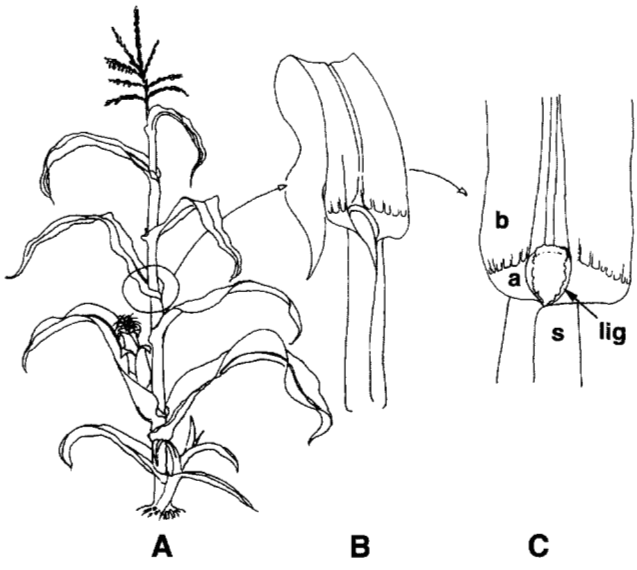

FIGURE 1.—A cartoon of an adult maize plant (A), a single leaf (B), and the ligular region under study (C). a, auricle; b, blade; s, sheath; lig, ligule.

Recessive mutations in either of the unlinked genes *lg1* or *lg2* remove the ligule and auricle. When introgressed into either the W23 or Mo17 background, *lg1* and *lg2* mutants can be distinguished in seedlings as well as in adult plants. *lg1* mutants have been extensively studied and do not develop auricle or normal ligule on any leaf (BECRAFT *et al.* 1990; SYLVESTER *et al.* 1990). Ligules are completely absent from about the first 10 leaves; however, on the upper five to 10 leaves a rudimentary ligule develops without accompanying auricle (BECRAFT *et al.* 1990; SYLVESTER *et al.* 1990). Although the mutant phenotypes of *lg1* and *lg2* are superficially similar, this report shows the unique phenotype specified by the *lg2-reference* (*lg2-R*) allele.

To better understand the biological role of *lg2*, we have used mosaic analysis, a successful tool to understand gene function [examples in maize include: *lg1-R* (BECRAFT *et al.* 1990; BECRAFT and FREELING 1991), *Kn1-O* (HAKE and FREELING 1986; SINHA and HAKE 1990), *D8-R* (HARBERD and FREELING 1989), *Tp1-R* and *Tp2-R* (DUDLEY and POETHIG 1993)]. Mosaic analysis requires the simultaneous somatic loss of gene function of a dominant allele of the gene of interest and of a known cell-autonomous marker. If the borders of the cell-autonomous marker sector perfectly coincide with the borders of the gene-of-interest's mutant phenotype, the wild-type product is considered cell-autonomous. If the borders do not coincide, the gene is considered cell-nonautonomous. In maize, such analysis has shown that *lg1* function in ligule development is cell-autonomous and is required in the cells that make the ligule and auricle (BECRAFT *et al.* 1990). In this paper, we show that *lg2* function is cell-nonautonomous in all tissues.

In addition to the mosaic analysis and description of the *lg2* mutant phenotype, we present a series of genetic experiments aimed at understanding the biological function of *lg2* and *lg1*. We show that both the *lg1-reference* (*lg1-R*) and the *lg2-R* alleles are genetic nulls. We also analyze various combinations of *lg1* and *lg2* alleles, and present our idea of how *lg1* and *lg2* act to induce a ligule and auricle.

MATERIALS AND METHODS

Generation of stocks used for mosaic analysis: The *lg2* gene is located at position 101 on chromosome 3L (BRINK 1933). The most useful cell-autonomous marker on that arm is *yellow10* (*y10*), located 13 map units distal to *lg2* (HARPER and FREELING 1993). In an anthocyaninless background, *y10* mutants have white kernels that produce white seedlings due to lack of carotenoids and chlorophyll. These seedlings die just after their second leaf has emerged, ~12 days from germination. The ligule phenotype (liguleless or wild type) can be scored before death.

To insure breakage events that uncovered *y10* coincidentally uncovered *lg2-R*, a large inversion on chromosome arm 3L (*Inv3a*) was used to switch the orientation of the dominant (wild-type) alleles $Lg2^+$ and $Y10^+$. Plants heterozygous for *Inv3a* were recognized by their phenotype of 18% pollen abortion and by suppression of recombination of markers located in or near the inversion (RHOADES and DEMPSEY 1953; BURNHAM 1962). Plants with 18% pollen abortion (*Inv3a/+*) were crossed to plants *lg2-R* $Y10^+$/*lg2-R y10*. Progeny were self-pollinated, and ears were selected that contain 25% white kernels. The white kernels were grown out and scored for their *lg2* phenotype. At this point, ears that segregated for the inversion were distinguished from ears that do not by the presence or absence of recombination as observed in the white progeny. Nonwhite (yellow) seeds from ears in which all white progeny had *lg2-R* phenotype (indicating no recombination) were used for generation of mosaics. These yellow kernels segregate for useful *Inv3a*($Lg2^+$ $Y10^+$)/*lg2-R y10* and useless *Inv3a*($Lg2^+$ $Y10^+$)/*Inv3a*($Lg2^+$ $Y10^+$) in a ratio of 2:1.

To control for any possible adverse effects of the inversion, *lg2 y10*/ $Lg2^+$ $Y10^+$ stocks were also used to generate mosaics. Stocks were regenerated by self-pollinating, and selecting ears in which the white kernels grew into phenotypically *y10 lg2-R* seedlings. Recombination of 13% was detected in these lines; thus, ~4.8% of X-rayed yellow seeds could have produced confounding *y10* $Lg2^+$ sectors. About 29% could have generated informative *y10 lg2* sectors. A stock of *Inv3a*/*lg2 y10* was generously given by Dr. SCOTT POETHIG, University of Pennsylvania. Additional lines were made using *lg2-R*, *y10* and *Inv3a* provided by the Maize Genetics Cooperation Stock Center, University of Illinois at Urbana.

Generation and growth of mosaics: Somatic sectors of genotypically *lg2-R/deletion* in otherwise wild-type leaves were generated by X-ray-induced chromosomal breakage in four separate experiments (two in the summer, grown in the University of California Bay Area Research and Extension Center, and two in the winter, grown in soil bed greenhouses in Albany, CA). Kernels were imbibed for 48 hr in the dark on a sand bench in a greenhouse or for 24 hr on wet paper towels in the light in a greenhouse before irradiation. Imbibed kernels were given 1000 rad of X-rays through a 0.35-mm Cu filter, from a Philips Model RT250 X-ray machine running at 225 kV. We preferred the 24-hr imbibition because the kernels were easier to plant without damage after irradiation.

Analysis of mosaics: Leaves that contained sectors were collected when fully expanded, from 4 to 11 weeks after irradiation. Sectors were accurately drawn on a standardized leaf cartoon, and leaf number, sector length, width, position,

shape, and special features were recorded. To determine which mesophyll layers the sector occupied, hand cross-sections were made of all sectors in the blade just above the auricle and of many sectors in the sheath just below the ligule. These were mounted in water and observed. In almost all cases, the tissue layers demonstrating loss were the same in the blade and sheath in a single-sectored leaf. Thus, only cross-sections made in the blade directly above the auricle are reported here. The sectors in cross-section were drawn on a standardized cartoon, with special attention to the relation of the sector boundaries and veins. To determine which, if any, epidermal layers the sector occupied, small strips of epidermis or small sections of leaves were mounted in water between a slide and coverslip. Because guard cells are the only chlorophyll-containing cells in the epidermis (sectors in the epidermis alone are not visible to the naked eye), only epidermis near white mesophyll was scored and recorded. Cross sections, epidermal peels, and whole mounts were visualized by epi-fluorescence microscopy through a Zeiss microscope using a 395- to 440-nm band path excitation filter with a long pass 470-nm barrier filter. At this excitation wavelength, green *Y10*$^{+}$ (wild-type) tissue autofluoresces red, while white *y10* tissue appears clear. Because a sector almost never simply occupies all layers of the mesophyll along the sector's lateral dimension, the transverse dimension of the leaf was divided into five layers from adaxial to abaxial: the adaxial epidermis (single cell layer), upper mesophyll (several cell layers), middle mesophyll (represented by the vasculature and the very middle mesophyll cell layer), lower mesophyll (several cell layers), and the abaxial epidermis (single cell layer) (LANGDALE *et al.* 1989). The epidermis originates from the L1 layer in the meristem. The upper, middle and lower mesophyll originate from the L2 layer of the meristem (FREELING and LANE 1994, and references therein). For simplicity, sectors were grouped as "L1 + L2," or "L2 only." White sectors present only in the L1 do exist, but are not visible by eye because only the guard cells of the epidermis have chloroplasts.

Several sectors were found on adult leaves in which the adaxial and abaxial epidermis were not the same genotype. These sectors had white mesophyll, so the white epidermis could be a cell layer invasion from L2 to L1, or the sector could have been present only in the upper or lower meristem flank, giving rise to only one white epidermis, followed by cell layer invasion from L1 to L2 to generate the white mesophyll. In addition, about half of the sectors at the margin of the blade had white mesophyll and green epidermis, suggesting that the leaf margin may not always be L1.

Dosage analysis of *lg2*: The *TB-3Lg* translocation was used to generate the genotype *lg2-R/deletion* (BECKETT 1978). *TB-3Lg* is the seventh *TB* translocation on chromosome *3* and is thus labeled "g." This designation is not related to a liguleless gene, and thus, we have dropped the g for clarity. Figure 2 shows the crosses made to observe *lg2-R* hemizygotes (hypoploids) and *lg2-R* homozygotes as siblings. Several plants from a *TB-3Lg* stock were crossed as females to *lg2-R/lg2-R* males. Several progeny from each cross were crossed as males to *lg2-R/lg2-R* females. It was assumed that males heterozygous for the TB-3Lg would generate the characteristically short hypoploids. The resulting families of progeny were planted in the field, and families that segregated tall *lg2* mutants (~50%, representing *lg2-R* homozygotes) and short *lg2* mutants (non-Mendelian ratios, representing *3*L hypoploids) were selected for further analysis. A few representative short *lg2* mutant plant were checked for pollen abortion and were found to shed 50% aborted pollen indicating that they were hypoploids. Likewise, a few tall *lg2* mutant plants were confirmed to shed normal pollen, indicating that they had a complete

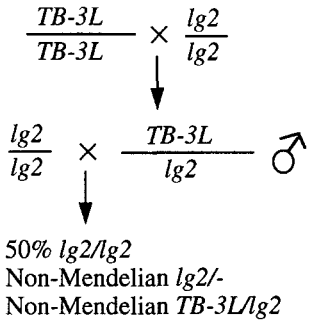

FIGURE 2.—Crosses for dosage analysis of *lg2*. *TB-3L* $^{+}$*/lg2* was crossed as a male to *lg2-R/ lg2-R*. The cross was set up this way to compare *lg2-R/ lg2-R* siblings to *lg2-R/ −*. Hypoploids of *TB-3L* are runt, so *lg2-R/ lg2-R* homozygotes could be distinguished from *lg2-R/ −* hypoploids. The *TB* translocation used was *TB-3Lg*. We have dropped the g here to avoid confusion (see MATERIALS AND METHODS).

chromosome complement. Several families segregating these genotypes were grown out in each of three summers for comparison. Liguleless plants were carefully evaluated several times each as seedlings, as adults, and during and postflowering. All phenotypically *lg2* mutant seedlings were labeled indicating the leaf on which auricle first developed. Stocks of *TB-3Lg* were obtained from Dr. JOHN FOWLER.

Dosage analysis of *lg1*: *TB-2Sb* plants were crossed individually as males to both *lg1-R/lg1-R* and to a *dwarf5* (*d5*) tester that is also located on chromosome arm 2S. If progeny of a *TB-2Sb* male are liguleless or dwarf respectively, the B-A chromosome was nondisjoining. Liguleless or dwarf progeny were observed, respectively, in non-Mendelian ratios. The *TB2-Sb* stock and *d5* tester were kindly provided by Dr. JACK BECKETT, University of Missouri, Columbia.

Double mutant analysis: The reference alleles, *lg1-R* and *lg2-R*, were used for this experiment. Because of the similarity in the phenotypes of *lg1* and *lg2* mutants, care was taken to ensure homozygosity of mutant alleles of both genes. The epidermal marker *glossy2* (*gl2;* 20 cM from *lg1*) was used to mark the chromosome carrying *lg1-R*. The crosses are presented in Figure 3. Liguleless, glossy progeny of cross 2 could have been heterozygous for *lg2-R* or homozygous for wild type. These progeny were self-pollinated and test-crossed to *lg2-R/lg2-R*. Progeny were planted from the self-pollination cross of an individual confirmed to be *lg1 gl2/lg1 gl2; lg2/+*. This progeny was 100% *lg1-R gl2/lg1-R gl2* and segregated 1:2:1 for *+/+*, *lg2-R/+*, *lg2-R/lg2-R*, as confirmed by test crosses to *lg1-R/lg1-R* and to *lg2-R/lg2-R*. The *lg1-R*, *gl2* and *lg2-R* alleles were originally from the Maize Genetics Cooperation Stock Center, University of Illinois at Urbana. These alleles had been introgressed four or more times into inbreds Mo17 and W23.

Allelism tests: All liguleless mutants used in this paper were crossed to both *lg1-R* homozygotes and to *lg2-R* homozygotes to confirm allelism. Progeny were grown in seedling flats and scored at 2–3 weeks old.

RESULTS

Phenotype of *lg2-R*: To date, all known mutations in the *lg2* gene are recessive and have a similar or identical phenotype. When introgressed six generations into the W23 inbred, the first and sometimes the second leaf completely lacked the ligule and auricle (Figure 4). Auricle and associated ligule developed at the margin

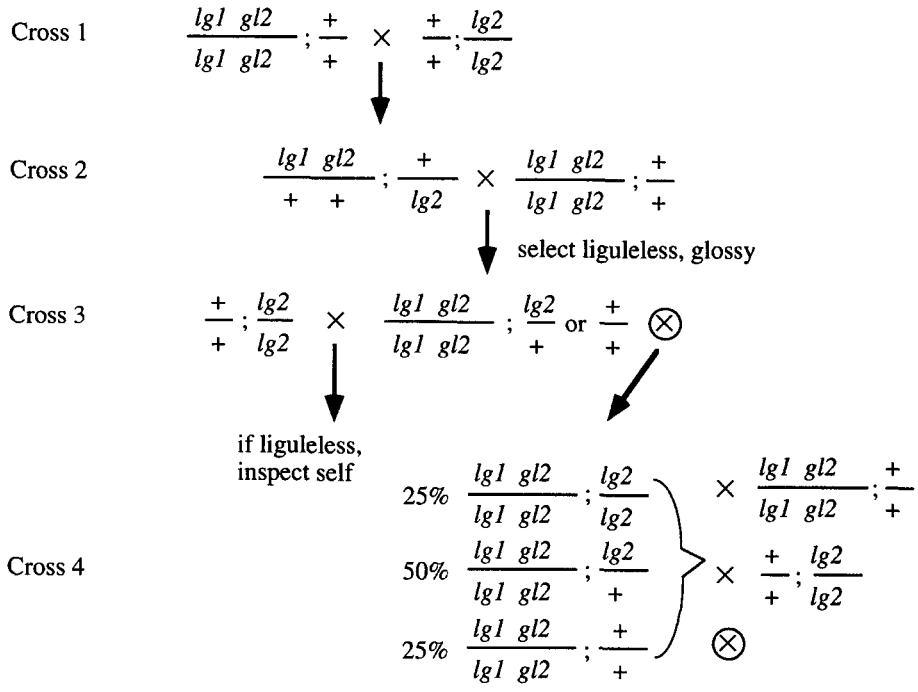

FIGURE 3.—Crossing scheme to generate *lg1-R lg2-R* double mutants. Progeny of the second cross were test crossed to *lg2-R* and self pollinated (cross 3). If the individual contained *lg2-R*, progeny of that self was grown, observed and testcrossed (cross 4).

of the third leaf. Often, these "auricle wedges" were displaced with respect to one another on the juvenile leaves (Figure 4F). Successive leaves gradually developed more ligule and auricle, until they looked almost wild type. This age-dependent expression can be seen by comparing Figures 4, C, F and I, and 6C.

Adaxial views of the ligular region on adult *lg2-R* mutant leaves revealed a region around the midrib where sheath has been displaced into the blade (not shown). Small patches of ligule could often be seen in the area near the midrib where no auricle develops. In general, the area on a *lg2* mutant leaf in which ligule and auricle developed occupied a greater portion of the leaf length. While ligule and auricle appeared more normal at the leaf margins of upper leaves, the area around the midrib almost never developed ligule and auricle.

Mutants of *lg2* also display the "liguleless" phenotype of upright leaves. While wild type leaves bend 45 degrees at the auricle causing the blade to lay out from the main axis of the plant, *lg2* mutant leaves do not bend at the blade sheath boundary. This manifests as an upright leaf attitude. Structural reasons for this bend are not precisely known. However, since even the upper leaves of *lg2* mutants are upright and they develop partial auricle, the bend may require a continuous auricle. This phenotype of upright leaves is also displayed in all *lg1* mutants.

Saturation of the phenotype: To our knowledge seven *lg1* mutant alleles and three *lg2* mutant alleles have arisen spontaneously or have been recovered from undirected mutagenesis experiments (Table 1). These mutants were discovered in the field by the distinctive upright stature due to the leaves not bending out at the auricle. We used two statistical methods of estimating the likelihood of missing a third gene that was capable of producing a recessive liguleless phenotype, if there were such a gene. Using the equation that describes the Poisson distribution ($P_i = e^{-m} \times m^i/i!$), we estimated the chance that if there were three genes, we missed one ($i = 0$; meaning zero alleles at a third gene). In this case, $P_0 = e^{-m}$ and $m = 5$ (m = the average number of alleles per locus). $P_0 = e^{-5} = 0.007$, and thus there is a 0.7% likelihood that we missed a third gene. This method, however, gives a poor estimate because the average number of alleles per gene ($m = 5$) cannot be precise. Thus, a second method was used to address the question: if there were exactly three genes in the genome capable of producing a recessive liguleless phenotype, what is the probability that out of 10 mutants, one of the three genes gave no alleles? This can be described by $3(2/3)^{10} = 0.052$, indicating that there is a 5% chance that we missed an allele of a third gene. The results of both methods indicate that the existence of another gene capable of giving a recessive liguleless phenotype is unlikely.

Several assumptions are made in these statistical calculations including that all loci are equally mutable, that transposable elements can transpose into any gene, and that all target genes are of the same length. While these assumptions are probably not true, these statistics gives us a very rough idea of the likelihood of the existence of a third liguleless gene.

Dosage analysis of *lg2-R* and *lg1-R*: For recessive alleles, comparison of homozygotes to hemizygotes can help distinguish between null and hypomorphic (underexpressing) alleles (MULLER 1932). To determine whether *lg1-R* and *lg2-R* are null or hypomorphic alleles, we used translocations of A chromosome arms to a su-

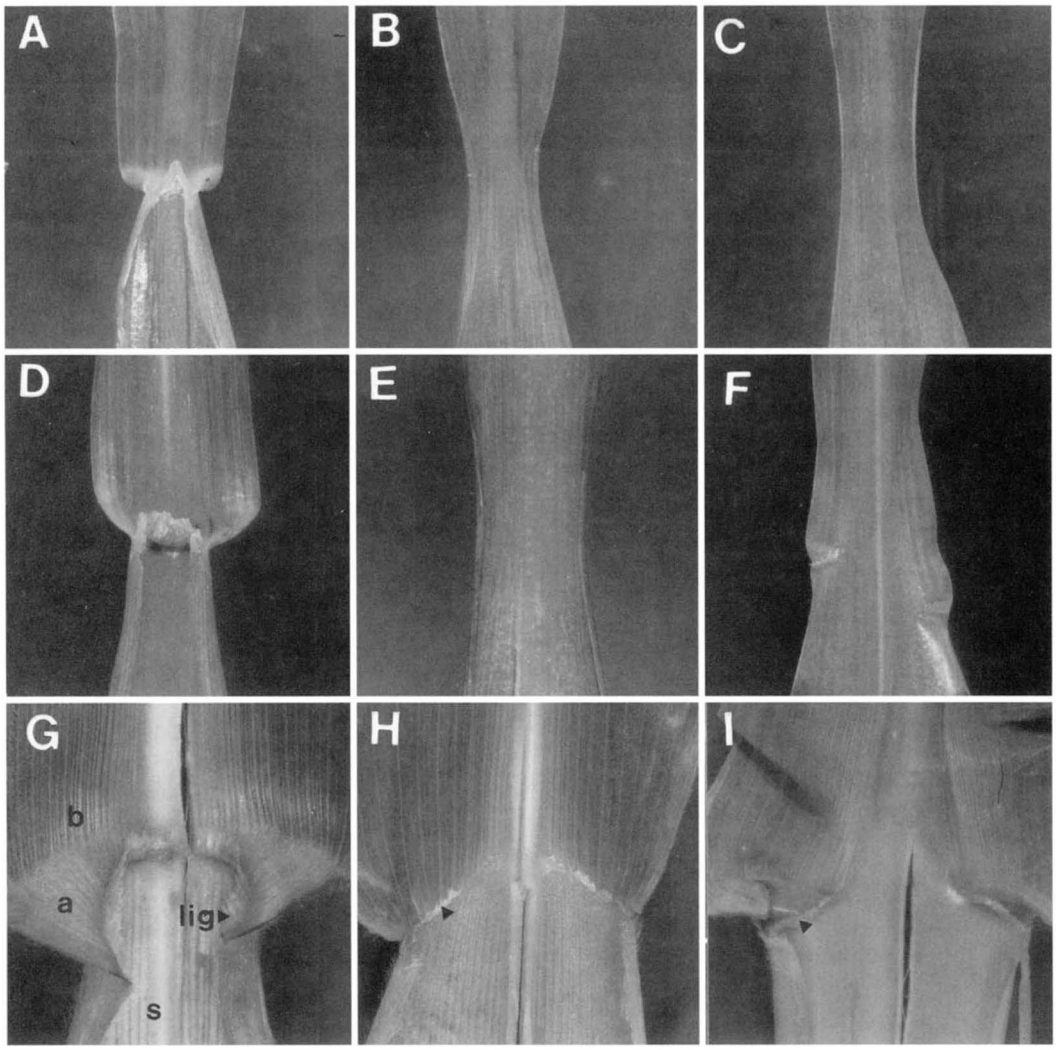

FIGURE 4.—Phenotype of wild type (A, D, and G), *lg1-R/lg1-R* (B, E, and H) *lg2-R/lg2-R* (C, F, and I). The ligular region of the first (A, B, and C), the fourth (D, E, and F) and the leaf subtending the primary ear (G, H, and I) are shown. Note that by the fourth leaf, the *lg1-R* (E) and *lg2-R* (F) mutant can be distinguished by the displaced auricle that develops in the *lg2-R* mutant. In the leaf subtending the primary ear, a rudimentary ligule can be seen in *lg1-R* (H, arrowhead), and partial auricle and rudimentary ligule can be seen in *lg2-R* (F, arrowhead). a, auricle; b, blade; s, sheath; lig, ligule.

pernumerary B chromosome (ROMAN 1947; BECKETT 1978). These B-A translocations transmit normally through the female. However, the B centromere frequently nondisjoins at the second mitotic microspore division of male gametophyte development, causing one sperm to carry a duplication of the translocated arm, and one to carry a deficiency. This process results in loss (hypoploidy) or gain (hyperploidy) of the translocated A arm in some progeny from crosses where the B-A translocation has been used as a male.

Due to the age-dependent expression of the *lg2-R* mutant phenotype, and the possibility of variation of this phenotype in different genetic backgrounds, it was considered necessary to compare *lg2-R* homozygotes (*lg2-R/lg2-R*) to hemizygotes (*lg2-R/deletion*) segregating in the same family. All phenotypically *lg2-R* mutant plants were compared at many stages throughout development. Plants hypoploid for chromosome arm 3L are short, so *lg2-R* homozygotes and hemizygotes could be distinguished by a liguleless-independent criteria during development. In addition, several of the short *lg2-R* plants were checked and confirmed to shed 50% aborted pollen, indicating that they were hypoploids. Several tall *lg2-R* siblings were confirmed to shed normal pollen, indicating that they contained a full chromosome complement.

Both the *lg2-R* hemizygotes (*lg2-R* hypoploids) and the *lg2-R* homozygotes displayed variability in the leaf on which ligule and auricle first developed (some on the second, some on the third). Both classes of lg2-R mutants showed the age-dependent expression of the phenotype discussed above. However, at each stage in development, no difference in the ligule and auricle was observed between leaves of *lg2-R/lg2-R* individuals and of *lg2-R/deletion* individuals. Thus, the *lg2-R* allele meets the criteria for being a "genetic" null (MULLER 1932).

Dosage analysis was also performed on *lg1-R*. When compared at many stages throughout development, the *lg1-R* hypoploids (*lg1-r/deletion*) appeared no different than their *lg1-R/lg1-R* progenitors with respect to ligule and auricle. Particular care was taken to score the presence of the rudimentary ligule in the upper leaves. This structure was observed in all *lg1-R* hypoploids. Therefore, the *lg1-R* allele also meets the criteria for being a genetic null.

TABLE 1

Mutant alleles of *lg1* and *lg2* that have arisen spontaneously or have been recovered from undirected mutagenesis

Allele	Source	Mutagen
lg1-R	EMERSON (1912)	Spontaneous
lg1-brink	BRINK (1933)[a]	Spontaneous
lg1-128	BRIGGS	*Mutator*
lg1-340	BRIGGS	*Mutator*
lg1-656	BRIGGS	*Mutator*
lg1-m1	DELLAPORTA and MORENO	*Ac*
lg1-m2	DELLAPORTA and MORENO	*Ac*
lg2-R	BRINK (1933)	Spontaneous
lg2-rb	FREELING	*Mutator*
lg2-neuffer	NEUFFER	EMS

Mutant alleles are indicated with their source and mutagen, if known. The absence of a date in the source column denotes unpublished alleles from Dr. STEVE BRIGGS, Pioneer Hi Bred; Drs. STEVE DELLAPORTA and MARIA MORENO, Yale University; or Dr. GERRY NEUFFER, University of Missouri.

[a] Lost.

Double mutants and other combinations of *lg1-R* and *lg2-R* alleles: Double mutants were constructed as described in Figure 3. A plant of genotype *lg1-R/lg1-R; lg2-R/+* was self-pollinated and 28 of the resulting progeny were carefully observed throughout development and test-crossed to determine their genotype. Scoring the *lg2-R* test crosses showed Mendelian segregation of *lg2-R:* seven plants were *lg1-R/lg1-R; +/+*, 15 plants were *lg1-R/lg1-R; lg2-R/+*, and six plants were *lg1-R/lg1-R; lg2-R/lg2-R*. Constructing the double mutants in this way allowed for the comparison of double mutants to single *lg1-R* mutant siblings in the same genetic background. All leaves of the 28 plants lacked auricle. As seedlings, these plants looked identical to each other, lacking ligule and auricle on all of the first eight to 10 leaves. This phenotype is typical of the *lg1-R* mutant and suggests a simple additive phenotype in the lower leaves. However, the phenotypes of the adult leaves from the primary ear leaf to the flag leaf fell into three distinct classes. All of the *lg1-R/lg1-R; +/+* plants had a rudimentary ligule on all of the upper leaves. The blade-sheath boundary appeared distinct on these plants; the sheath was less green than the blade, and the adaxial sheath was shiny, while the blade appeared dull, as is typical of the *lg1-R* mutant. In contrast, the six double mutant *lg1-R/lg1-R; lg2-R/lg2-R* plants had a novel phenotype of absolutely no ligule on any leaf and a blade-sheath boundary that was not distinct (Figure 5). That is, the amount of chlorophyll in the blade and sheath in the region of the boundary was very similar, and the shiny appearance of the adaxial sheath extended into the blade. The *lg1-R/lg1-R; lg2-R/+* plants fell into two distinct classes. Five plants had a typical *lg1-R* phenotype, while 10 plants had a novel phenotype of no ligule at all on any leaf, but still maintained a distinct blade-sheath boundary.

Although the lower leaves of the double mutants displayed an additive phenotype, a synergistic interaction between the *lg1-R* and *lg2-R* mutant phenotypes was apparent from the lack of both a rudimentary ligule and a distinct blade-sheath boundary in the upper leaves. This suggests *lg1* and *lg2* are in a common biological circuit of action. The novel phenotype of some of the *lg1-R/lg1-R; lg2-R/+* sibs of double mutants supports this conclusion.

Data supporting an interaction between *lg1* and *lg2* were obtained from observing the progeny of test crosses of the double mutants to *lg1-R/lg1-R* and to *lg2-R/lg2-R* (see Figure 3, cross 4). Progeny of these test crosses were grown to flowering and compared to their single homozygous mutant mothers (*lg1-R/lg1-R; lg2-R/+* compared to *lg1-R/lg1-R; +/+*, and *lg1-R/+; lg2-R/lg2-R* compared to *+/+; lg2-R/lg2-R*). Surprisingly, the adult leaves between the leaf subtending the primary ear and the flag leaf of 10 *lg1-R/lg1-R; lg2-R/+* individuals observed displayed a more severe phenotype than 10 *lg1-R/lg1-R; +/+*, individuals (no ligule on any leaf, but a distinct blade-sheath boundary was maintained) and the same leaves of 10 *lg1-R/+; lg2-R/lg2-R* individuals displayed a more severe phenotype than 10 *+/+; lg2-R/lg2-R* individuals (less ligule and auricle developed) (Figure 6). Both homozygote-heterozygote combinations were still less severe than the double mutant. This sensitivity to each other's dose implies an interaction between *lg1* and *lg2* function. Specifically, LG2 must be expressed in *lg1-R/lg1-R* plants, and LG1 must be expressed in *lg2-R/lg2-R* plants.

Thus, the synergistic double mutant phenotype suggests that the products of the *lg1* and *lg2* genes act in the same pathway, and the reciprocal dosage sensitivity suggests that the products of *lg1* and *lg2* genes may interact. Whether their interaction is direct or indirect has not been tested here.

Mosaic analysis of *lg2*: Mosaic analysis was performed to determine the autonomy of the *lg2-R* phenotype. X-rays were used to induce the loss of chromosome arm 3L ($Lg2^+$ $Y10^+$) in a single cell early in development of the leaf or in the meristem. Simultaneous loss of $Lg2^+$ and $Y10^+$ created a visible white clone of genotypically *lg2-R y10/deletion* cells on fully expanded green leaves. Three different genotypes were used for the experiment (see Figure 7, and MATERIALS AND METHODS).

Combining the four mosaic analysis experiments, 1630 X-rayed seeds of genotype *Inv3a*($Lg2^+$ $Y10^+$)*/lg2 y10* gave 42 sectored plants with a total of 72 sectored leaves, 1798 X-rayed seeds of genotype $Lg2^+$ $Y10^+$*/lg2 y10* gave 81 sectored plants with a total of 136 sectored leaves, and 2689 X-rayed seeds of genotype $Lg2^+$ $Y10^+$/ $Lg2^+$ *y10* (controls) gave 84 sectored plants with a total of 142 sectored leaves. Thus, a total of 208 sectored experimental leaves, and 142 sectored control leaves were analyzed.

We checked a number of parameters to make sure

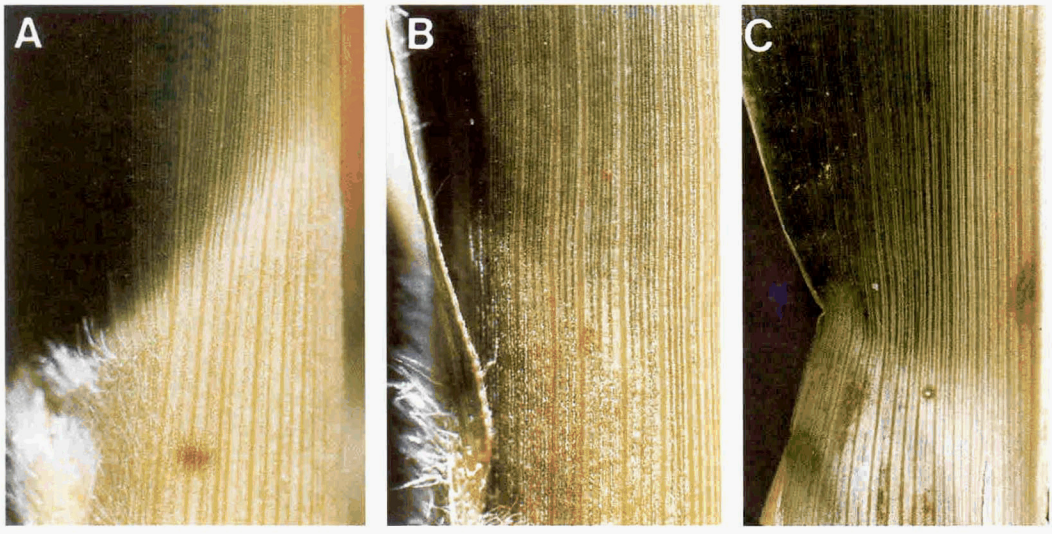

FIGURE 5.—Phenotype of the *lg1-R lg2-R* double mutants in the upper leaves. (A) Abaxial view of *lg1-R/lg1-R* plant next to (B). *lg1-R/lg1-R, lg2-R/lg2-R* sibling (left). (C) Adaxial view of a *lg1-R/lg1-R, lg2-R/lg2-R* leaf showing no ligule or auricle.

we were not missing a critical class of sectors that might reveal *lg2* autonomy. First, sectors were examined for tissue layer of chromosome loss, and scored as either "L2" or "L1 + L2" referring to the meristematic layer in which the loss occurred (see Table 2, and MATERIALS AND METHODS). Many sectors of both types were found. Second, when drawings of all the experimental sectors where superimposed, we confirmed that sectors were found across the entire lateral dimension of the leaf (midrib to margin). While sectors directly in the center of the midrib cannot be seen at the ligule, they can be inferred from the presence of a small white sector at the very tip of a leaf just to one side of the midvein. Such a sector is usually seen on a leaf that resides between two

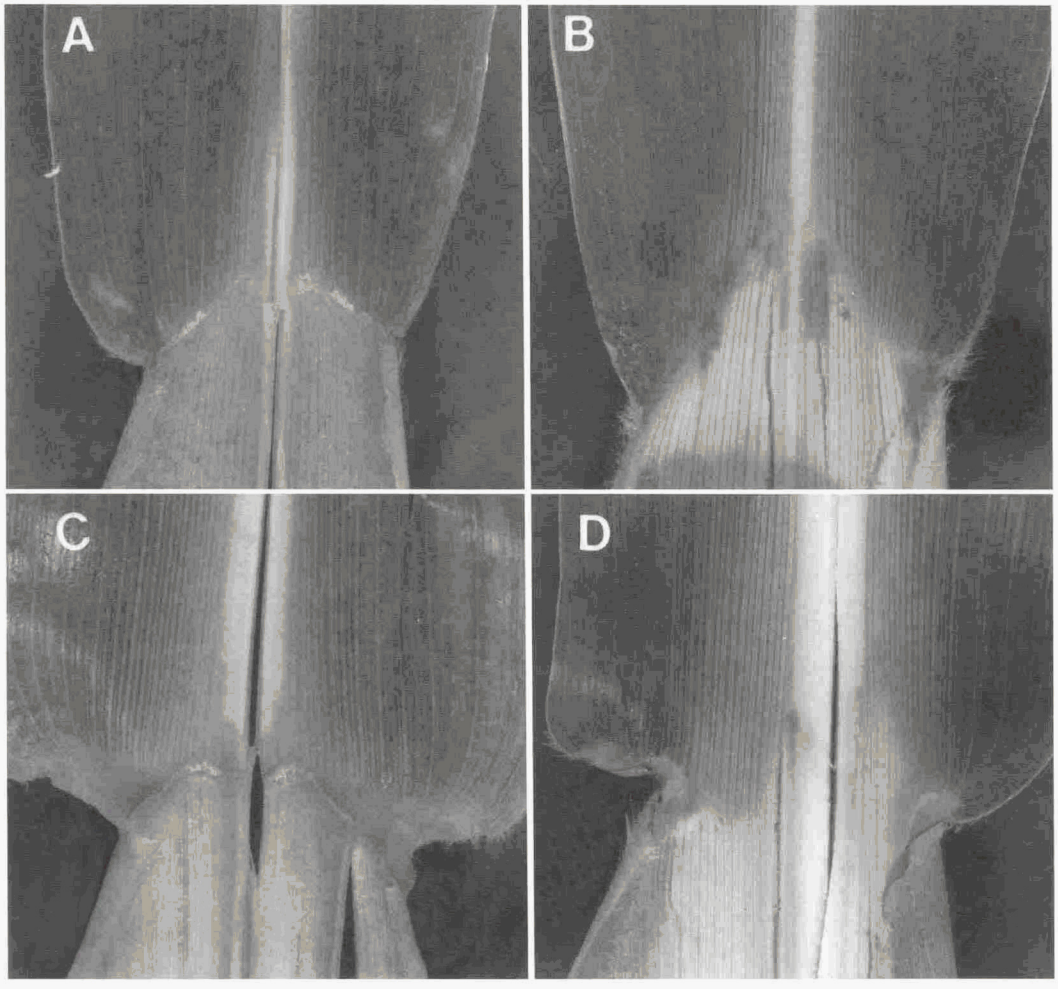

FIGURE 6.—The effect of gene dosage of *lg1* on the *lg2-R* mutant phenotype and of *lg2* on the *lg1-R* mutant phenotype. Leaves were taken from midway between the leaf subtending the primary ear and the flag leaf. Compare A (*lg1-R/lg1-R; +/+*) to B (*lg1-R/lg1-R; lg2-R/+*), and compare C (*+/+; lg2-R/lg2-R*) to D (*lg1-R/+; lg2-R/lg2-R*).

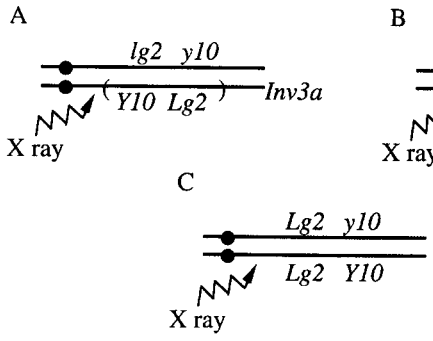

FIGURE 7.—Chromosome 3 configuration of plants exposed to X-rays to induce breakage. (A) Use of an inversion insures any loss of (*Y10 Lg2*) arm that uncovers *y10* will also uncover *lg2-R*. (B) Conventional heterozygote. Most arm losses will include both genes; however, a break between *Lg2* and *Y10* is possible. (C) Control chromosomes carry the wild-type allele of *lg2* on both homologues.

leaves that have sectors toward one of their margins. We observed several such sectors and inferred the presence of a sector at the center of the midrib. Third, the sectors ranged in width from less than a millimeter to 2.5 cm in width, representing up to one-quarter of the width of the leaf at the ligule. Fourth, sectors were also found on all leaves from the fourth leaf to the flag leaf. Fifth, both single leaf sectors (originating in leaf, or small meristem sectors) and multiple leaf sectors (sector originating and maintained in meristem) were found. Sixth, to examine the unlikely scenario of a focus of *lg2* action in the blade, 26 experimental sectors were examined that existed in the blade only and did not extend to the auricle (called "L2 blade" in Table 2). These were all L2 sectors and represented loss of the chromosome arm late in leaf development when the blade is differentiating basipetally (STEFFENSEN 1968; POETHIG 1984). This wide spectrum of sector types is of the expected range and suggested that an informative category was not missed.

No *lg2-R/deletion* sectors removed the ligule and/or auricle. The majority of both experimental (82%) and control (87%) sectored leaves showed no effect on the ligule or auricle (Figure 8). This result indicates that lack of a wild-type $Lg2^+$ allele in sectors has no effect on ligule or auricle differentiation, and therefore the lg2-R phenotype is cell-nonautonomous. Since we saw normal ligule and auricle development in sectors of both tissue layer categories "L2 only," and "L1 + L2," the phenotype is cell-nonautonomous in both the lateral (from midrib to margin) and transverse (adaxial to abaxial) dimensions of the leaf.

Eighteen percent of experimental and 13% of control sectored leaves showed some effect on ligule and/or auricle development. These "effects" were of several types: a small notch or reduction in the ligule somewhere within the sector but not coinciding with the borders of the white sector (60% of aberrant sectors), or an upward displacement of the upper auricle border on the distal (marginal) side of the sector (20%), or both (20%). Because these anomalies were seen in similar percentages in both experimental and control sectors, they cannot be attributed specifically to loss of the $Lg2^+$ allele.

TABLE 2

Number of type and sectors found in mosaic analysis of *lg2*

Genotype	Tissue layer of *Lg2*+ loss	Effect on ligule or auricle	Sectored plants	Sectored leaves
lg2 y10/Inv3a	L2	Wild type	22	36
	L2 blade	Wild type	0	0
	L1, L2	Wild type	5	14
	L2	Aberrant	6	9
	L2 blade	Aberrant	0	0
	L1, L2	Aberrant	9	13
lg2 y10/++	L2	Wild type	33	72
	L2 blade	Wild type	25	25
	L1, L2	Wild type	9	24
	L2	Aberrant	6	7
	L2 blade	Aberrant	1	1
	L1, L2	Aberrant	7	7
Controls				
y10/+	L2	Wild type	49	87
	L2 blade	Wild type	8	8
	L1, L2	Wild type	12	28
	L2	Aberrant	10	13
	L2 blade	Aberrant	0	0
	L1, L2	Aberrant	5	6

Total number of sectors and sectored leaves found in each of the three genotypes are shown. Sectors are divided by the tissue layer in which the chromosome arm loss was apparent, and by whether or not there was an effect on the ligule-auricle. See text for description of effects.

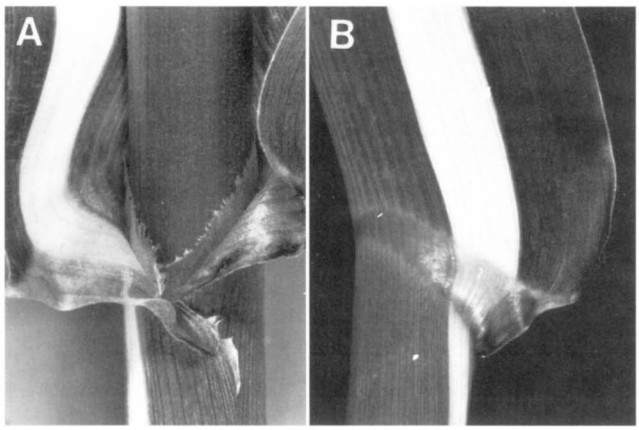

FIGURE 8.—A sector of *lg2-R y10*/− − on a *Lg2*$^{+}$ *Y10*$^{+}$/*lg2-R y10* leaf. Notice the normal ligule and auricle within the sector. (A) Adaxial. (B) Abaxial.

DISCUSSION

Saturation of liguleless mutant phenotype: We have probably identified the only two genes in maize capable of giving a recessive "liguleless" mutant phenotype. This phenotype is specifically the upright habit of the leaves caused by the lack or reduction of auricle, not the lack of ligule. Because of the intimate developmental association between the ligule and auricle, we expect any genes involved in the initiation or induction of ligule and auricle to have this phenotype. Saturation of the "liguleless" phenotype means that other genes involved in ligule and auricle initiation and development are not identifiable as liguleless mutants. These other genes are either required elsewhere in development (and would condition a different, perhaps lethal, mutant phenotype) or are duplicated. Since the maize genome is a putative ancestral allotetraploid (see MOORE *et al.* 1995), it is possible that there are duplicated genes with nondiverged functions involved specifically in ligule and auricle initiation and development. However, there are also only two genes identified in rice (see the Rice Genetic Newsletter, Vol. 9) that have liguleless mutant phenotypes: *lg* and *aur*. The genomes of rice and maize are generally collinear, but the rice genome is not duplicated in relation to other grasses (MOORE *et al.* 1995; PATERSON *et al.* 1995). The chromosomal locations of the maize *lg1* gene and the rice *lg* gene are syntenous (PRATCHETT and LAURIE 1994). Furthermore, these mutants have similar phenotypes. In addition, *aur* of rice has a similar mutant phenotype to *lg2* of maize, although it is not yet known if these are syntenous (M. MOONEY, this lab, unpublished data). This suggests that any duplicated copies of *lg1* and of *lg2* have diverged enough to provide functions different from those of *lg1* and *lg2*. Considering the evolutionary relatedness of maize and rice, we suggest that these two liguleless genes are the only ones in the grasses that can be identified by the recessive liguleless mutant phenotypes. This may indicate that among the many genes necessary to make a ligule and auricle, *lg1* and *lg2* may be the only genes uniquely required in ligule and auricle development. A mutant that lacked only the ligule and maintained the auricle is still possible, although this phenotype has never been seen in maize.

Other genes that play a role in ligule and auricle development will have to be identified in ways more inventive than random mutagenesis, such as the creation of suppressers of the liguleless phenotype or molecular methods to identify ligule-specific molecules. We may have observed the effects of other genes involved in ligule and auricle development in our mosaic analysis. There were some effects seen on the ligule and/or auricle in a small percentage of both experimental (18%) and control (13%) leaf sectors that cannot be attributed to *lg2* dosage. While these effects may simply be developmental aberrations, it is also possible that they may be the result of hemizygosity at a particular gene(s) involved in ligule and auricle development. Because it may be required elsewhere in development and have a lethal mutant phenotype, this gene(s) may show a liguleless phenotype only in sectors on a leaf. If it is linked to *lg2,* the low frequency of chromosome breakage events that led to effects on the ligule and auricle suggest that the gene(s) may be proximal to *y10* and *lg2*. Conversely, coincidental breaks of other chromosome arms may be responsible for these effects.

***lg1* and *lg2* wild-type functions are involved in ligule and auricle development:** Comparison of *lg1-R* or *lg2-R* homozygotes to hemizygotes shows that these alleles meet the criteria for genetic null alleles. These genetic results have since been corroborated with molecular analysis. In the case of *lg1-R,* molecular analysis has revealed that this allele has a deletion of *lg1* genomic sequences (L. HARPER, M. MORENO, R. KRUEGER, S. DELLAPORTA and M. FREELING, unpublished results). Additionally, an allele of *lg2* that has a deletion of genomic sequences has been found (J. WALSH and F. FREELING, unpublished results), and its phenotype is very similar, if not identical to that of the *lg2-R* allele. We conclude that the molecular lesions in both the *lg1-R* and *lg2-R* mutants result in null, loss of function alleles. Our results show that MULLER's 1932 guidelines (MULLER 1932) for analysis of allele types are still valid and valuable.

The mutant phenotypes of *lg1-R* and *lg2-R* are similar in that they both remove or reduce the ligule and auricle on the maize leaf. Since these mutants are recessive null alleles, the function of both wild-type genes must be in the development of the ligule and auricle. *lg1* function must be an absolute requirement for auricle development, for any ligule on the lower leaves, and for full length ligules on the upper leaves. *lg2* function must be required for full elaboration of ligule and auricle, but also for correct initiation and positioning of the ligule and auricle.

***lg1* and *lg2* act in a common circuit of action:** In lower leaves of the *lg1-R lg2-R* double mutant, a simple additive phenotype of no ligule or auricle is observed. This can be meaningless if two mutants have identical phenotypes (AVERY and WASSERMAN 1992), but we have shown that lg1-R and lg2-R homozygotes are readily distinguishable in their upper leaves (Figure 4). The synergistic phenotype apparent in the upper leaves of a *lg1-R lg2-R* double mutant suggests that LG1 and LG2 act in the same biological pathway. In addition, siblings of double mutants and progeny of test crosses revealed a general trend apparent in the upper leaves: the *lg1* null mutant phenotype is sensitive to the dose of the *lg2* gene, and the *lg2* null mutant phenotype is sensitive to the dose of the *lg1* gene. This implies that LG1 is expressed in the *lg2* null mutant, and that LG2 is expressed in the *lg1* null mutant. Both LG1 and LG2 must interact with other gene functions that produce the rudimentary ligule and auricle in the *lg1* and *lg2* null mutants. To carry the implications even further, the dosage sensitivity may suggest that LG1 and LG2 may act temporally close to each other in the development of the ligule and auricle. If a biochemical step involving a cascade or amplification of a signal would separate the actions of *lg2* and *lg1,* we would not have observed this dosage effect. Additionally, the reciprocal dosage effects are expected if the LG1 and LG2 proteins require a fixed stoichiometry for function.

Is there a default mechanism that produces rudimentary ligule on the upper leaves of *lg1* mutants? In contrast to the completely liguleless and auricleless leaves initiated early in development of *lg1-R* and *lg2-R* mutants, both mutants produce either some ligule, or ligule and auricle, on leaves developed later. In wild-type maize, there is little detectable morphological difference between ligules and auricles of all leaves. However, in many grasses, adult leaves produce more elaborate ligules and auricles than juvenile leaves (CHAFFEY 1985). In an extreme example, CHAFFEY identified nine grass species that had membranous ligules on lower leaves, and veined ligules on the upper leaves (CHAFFEY 1985). This observation suggests that mechanisms may exist in the grasses for augmenting ligule and auricle differentiation in an age-dependent manner. If this is the case, the phenotype of the *lg1* null mutant might have uncovered this otherwise masked program in maize. The rudimentary ligule in adult leaves of the *lg1* null mutant may be the product of this "adult ligule elaboration" program.

There is another possible explanation for the rudimentary ligule in the upper leaves of *lg1* null mutants. The *lg* gene of rice and the *li* gene of barley are collinear with the *lg1* gene of maize (PRATCHETT and LAURIE 1994). However, preliminary observations show that the mutant phenotype in rice and barley is completely liguleless on all leaves, even the upper leaves (PRATCHETT and LAURIE 1994; M. MOONEY, unpublished observations). This presents the possibility that in maize, duplicated and diverged copies of *lg1* may be present and play a role in ligule development in the upper leaves. The same may be true for *lg2.* However, *aur* in rice and *lg2* in maize have nearly identical mutant phenotypes including the extensive auricle development in upper leaves. This datum does not support the duplicated and diverged gene explanation of the maize *lg2* phenotype.

These two possibilities are not mutually exclusive; the duplicated and diverged copies of *lg1* and *lg2* may be under the control of a general "adult ligule elaboration" program. In any case, the total lack of rudimentary ligule in the *lg1-R lg2-R* double mutant strongly suggests that an interaction of *lg1* and *lg2* wild-type function is involved in rudimentary ligule and auricle formation. If the rudimentary ligule in a *lg1* null mutant is due to the action of a duplicated and diverged copy of *lg1* (and/or *lg2*), this copy(s) must interact with both LG1 and LG2.

***lg2* function specifies the position of ligule-auricle induction:** The mosaic analysis showed that mutant sectors of *lg2-R y10* on a wild-type leaf produce wild-type ligule and auricle. *lg2* function is thus cell-nonautonomous, which suggests that either LG2 itself moves or induces another molecule to move, over regions at least as large as the widest sector (a quarter of the width of the leaf). When a result of cell-nonautonomy is found in a mosaic analysis, one can ask whether there is a focus of gene action. If there were a focus of *lg2* function, we would expect to find a subset of *lg2-R y10* sectors in a particular region of the leaf would remove ligule and auricle not only within the sector, but also outside the sector. The region of the leaf in which such sectors were found would represent the focus of *lg2* gene action. We found no such sectors in our *lg2* mosaic analysis. Because sectors were found across the whole lateral dimension of the leaf, we conclude that there is no focus of *lg2* function.

A cell-nonautonomous function with no focus of action may indicate the presence of a uniform field of *lg2* action across the whole lateral dimension of the leaf. The *lg2* null mutant phenotype suggests the role of this developmental field may be to correctly position the initiation and progression of the preligule band.

The role of *lg1* and *lg2* in specification of the blade-sheath boundary: The blade-sheath boundary is thought to be established very early, by the time a leaf primordium is in its second to third plastochron (SYLVESTER *et al.* 1990; FREELING 1992). Our observations showed that *lg2-R* mutants produce ligule and auricle in small patches in a wider area compared to wild type, indicating that a normal blade-sheath boundary is not established in the *lg2-R* mutant. SNYDER and BERTRAND-GARCIA (1993) also observed a disturbed blade-sheath boundary in an analysis of another *lg2* allele, *lg2-2757* (obtained from a directed mutagenesis). In scanning electron micrographs of mature leaves, they observed blade and hair cells below the

ligule, cells types that were always above the auricle in wild-type siblings. These data further indicate the inability of *lg2* mutants to form a normal blade-sheath boundary, suggesting that the wild-type *lg2* function acts very early in leaf primordia development, and may interact with molecules that specify the blade-sheath boundary.

BECRAFT and coworkers (1990) state that the blade-sheath boundary in the *lg1-R* mutant is "less distinct" than that of wild type. The *lg2-R* null mutant blade-sheath boundary is more disturbed than that of the *lg1-R* null mutant. However, the double mutant lacks almost all visible markers of a mature blade-sheath boundary. This indicates that the *lg1* and *lg2* wild-type functions may interact in the formation of a normal blade-sheath boundary.

Roles of *lg1* and *lg2* function in ligule and auricle induction and development: In a mosaic analysis of *lg1-R* mutant sectors on wild-type leaves, BECRAFT and coworkers (1990) established that *lg1* function is in most cases cell-autonomous, and is required in the epidermis for ligule development and in the mesophyll for auricle development. BECRAFT and coworkers did find that absence of the *lg1* gene in small sectors within the auricle mesophyll did not affect internal auricle histology. This was the only case of cell-nonautonomy reported. BECRAFT and FREELING (1991) presented a model where a signal "organizes development of the ligular region." They also found an unexpected phenomenon: the ligule and auricle "restarted" on the marginal side of all *lg1* sectors. This led them to propose the make-ligule-make-auricle signal cannot traverse *lg1-R* mutant sectors. Thus, LG1 is required not only to act on this signal but to propagate it as well. In addition, 40% of the time, reinitiated ligule and auricle were displaced downward, suggesting that the reinitiated ligule-auricle differentiated later in time than that closer to the midrib (BECRAFT and FREELING 1991). This led them to propose that the make-ligule-make-auricle signal emanates from near the midrib and moves outward toward the margin preceding the directional development of the preligular band and actual ligule outgrowth. LG1 does not appear to be involved in initiation of the make-ligule-make-auricle signal (BECRAFT *et al.* 1990; BECRAFT and FREELING 1991). *lg1* function is thus in the reception end of the ligule/auricle induction pathway.

As discussed above, the products of *lg1* and *lg2* act in a common circuit of action, and *lg2* function behaves in a cell-nonautonomous manner. One could ask whether LG2 represents the make-ligule-make-auricle signal. LG2 cannot be the only signal, because the ligule and auricle that develop in the *lg2-R* null mutant would not be expected if the make-ligule-make-auricle signal were absent. Instead, it is likely that the field of *lg2* function acts to restrict the make-ligule-and-auricle signal to a thin line across the lateral dimension of the primordium. Without the *lg2* field, the signal is still sent but induces ligule and auricle in a broader, more disorganized line. In wild-type plants, once the *lg2* field is established and the make-ligule-make-auricle signal is sent, *lg1* function acts to interpret and propagate this signal. We therefore expect to find that *lg2*'s function acts before *lg1*'s.

We do not yet know what factors are involved in initiating the process of ligule and auricle development. A clue is provided by several dominant leaf mutants that have ectopic ligule and auricle in novel places on the leaf. Among these are dominant mutants that ectopically express the homeobox genes *knotted1* (*kn1*), *rough sheath1* (*rs1*) and *liguleless3* (*lg3*) (VOLLBRECHT *et al.* 1991; SCHNEEBERGER *et al.* 1995; FOWLER *et al.* 1996, respectively), and several uncloned genes, *rolled1* (*rld1*), *hairy sheath frayed1* (*hsf1*), and *lax midrib1* (*lxm1*) (see FREELING 1992). Unlike *lg1* and *lg2* mutants, these mutants actually change the shape and/or position of the blade-sheath boundary. However, ligule and auricle induction simply follows these aberrant shapes. It is possible that the dominant mutants change the shape of the ligule-auricle developmental field within the leaf primordia. Then, the *lg2* and *lg1* genes perform their normal function within these new parameters. Mutant phenotypes of these dominant mutants, especially *rs1* and *lg3* that strikingly effect the ligule auricle region specifically (BECRAFT and FREELING 1994; FOWLER and FREELING 1996), lend support to the concept of a ligule-auricle developmental field within the leaf primordia.

We especially thank BARBARA KLOECKENER-GRUISSEM, MARK MOONEY, JUSTINE WALSH, and JOHN FOWLER for stimulating discussion and criticism during the course of this research. We thank BARBARA KLOECKENER-GRUISSEM, MARK MOONEY, JUSTINE WALSH, RANDALL TYERS, LYNNE JESAITIS, MIKE SCANLON and BARBARA LANE for critically reading this manuscript. We especially thank MARK MOONEY for drawing Figure 1 and for providing useful discussions relating maize to other grasses. We thank the Maize Coop, JACK BECKETT, JIM BIRCHLER, and SCOTT POETHIG for providing strains and advice. We thank KARL BROMAN for lessons in statistics. We thank the Clarinet Cafe for their stimulating lattes. We thank JOANNE WATKINS, BARBARA ROTZ and THOM KOUPAL for providing outstanding care of our plants. This work was supported by a grant from the U.S. Department of Energy (DOE grant DE-FG03-91ER20028).

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Communicating editor: J. A. BIRCHLER